Universidad de Córdoba
FACULTAD DE CIENCIAS
Departamento de Química Analítica

Análisis orientado y global en metabolómica vegetal mediante espectrometría de masas acoplada a técnicas cromatográficas

María Molina Calle

Tesis Doctoral
Córdoba 2017

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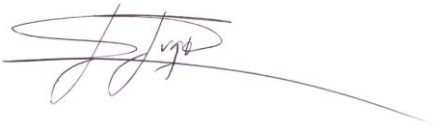
Los Directores,



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Trabajo presentado para optar al grado de
Doctora en Ciencias, Sección Químicas

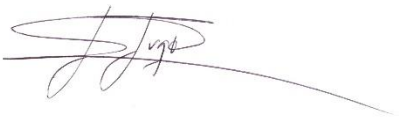
Fdo. María Molina Calle
Licenciada en Química

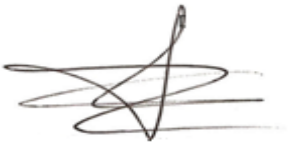
María Dolores Luque de Castro, Catedrática de Universidad, y **Feliciano Priego Capote**, Profesor Contratado Doctor, ambos del Departamento de Química Analítica, Facultad de Ciencias, Universidad de Córdoba, en calidad de Directores de la Tesis Doctoral presentada por la Licenciada en Química María Molina Calle, con el título "Análisis orientado y global en metabolómica vegetal mediante espectrometría de masas acoplada a técnicas cromatográficas",

CERTIFICAN:

Que la citada Tesis Doctoral se ha realizado en los laboratorios del Departamento de Química Analítica, Facultad de Ciencias, Universidad de Córdoba y que, a su juicio, reúne los requisitos necesarios exigidos en este tipo de trabajos.

Y para que conste y surta los efectos pertinentes, expiden el presente certificado en Córdoba, 6 de marzo de 2017.





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TÍTULO DE LA TESIS: Análisis orientado y global en metabolómica vegetal mediante espectrometría de masas acoplada a técnicas cromatográficas

DOCTORANDA: María Molina Calle

INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

La doctoranda María Molina Calle manifestó en el planteamiento de la tesis su interés por la metabolómica vegetal, por lo que la decisión de enfocar su investigación en esta área de trabajo del grupo fue consensuado por los directores y la doctoranda, que ha puesto de manifiesto durante el desarrollo de la investigación su vocación y lo acertado de la elección.

Los tres aspectos que ha abarcado su tesis enmarcada, por tanto, en la metabolómica vegetal han sido: (i) caracterización de residuos, como la piel de la naranja, basada en una identificación exhaustiva de sus componentes y de las mejores condiciones para su almacenaje; (ii) estudio en profundidad de una planta poco estudiada, como es la estevia rebaudiana, que requería un conocimiento profundo de su composición y de las mejores condiciones de adaptación a nuestra climatología; (iii) investigación comparada de un condimento común en España, como es el ajo, y de los productos derivados, que han incrementado el interés y el rendimiento económico de este bulbo y que ha servido para poner de manifiesto los cambios tan significativos que ocurren durante el proceso de fermentación que dan lugar al ajo negro a partir del fresco. Todo ello ha servido para poner de manifiesto la capacidad de la doctoranda para enfrentarse a cualquier reto, su pericia para resolverlo y la formación indiscutible que ha adquirido en todas las áreas en las que se ha involucrado.

El cuerpo de la Tesis lo conforman 10 artículos, cada uno de los cuales constituye un capítulo de la Memoria, que se ha dividido en 3 partes o bloques en función de la materia a la que se ha dedicado la investigación.

La parte A está constituida por 3 artículos/capítulos de los que el primero es una revisión bibliográfica de la materia sobre la que se investiga en los otros dos: la caracterización de residuos de la industria de zumos de cítricos. Estos dos capítulos constituyen una sólida aportación a la composición de la piel de naranja, su forma de conservación y la

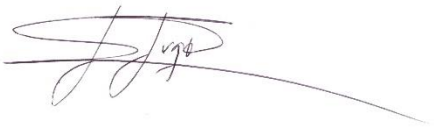
cuantificación de los compuestos más abundantes y característicos de este material: los flavonoides.
El bloque B lo constituyen también 3 artículos en los que se ha profundizado en la composición de la estevia rebaudiana, tanto en los esteviósidos como en compuestos poco estudiados en esta planta, como los compuestos polares, con identificación y cuantificación de compuestos desconocidos de la planta en cuestión.
La última parte de la Memoria la forman 4 artículos dedicados al estudio de los componentes del ajo fresco y del ajo negro y a la comparación entre ambos, tanto en lo que se refiere a los compuestos volátiles, como a los polares.

Consideramos que la investigación desarrollada y recogida en la Memoria reúne los requisitos de originalidad, innovación y calidad, y autorizamos la presentación de la Tesis Doctoral de María Molina Calle.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 6 de marzo de 2017

Firma de los directores



Fdo.: M. D. Luque de Castro

Fdo.: F. Priego Capote

INFORME SOBRE EL FACTOR DE IMPACTO DE LAS PUBLICACIONES DE LA TESIS

TÍTULO DE LA TESIS: ANÁLISIS ORIENTADO Y GLOBAL EN METABOLÓMICA VEGETAL MEDIANTE ESPECTROMETRÍA DE MASAS ACOPLADA A TÉCNICAS CROMATOGRAFÍCAS

DOCTORANDA: MARÍA MOLINA CALLE

PUBLICACIÓN	FI	DECIL/CUARTIL
Ultrasound-assisted emulsification−extraction of orange peel metabolites prior to tentative identification by LC−QTOF MS/MS. **Talanta**, 2015, 141, 150−157.	3.545	Q1 11/74 Chemistry, Analytical
Development and application of a quantitative method for determination of flavonoids in orange peel: Influence of sample pretreatment on composition. **Talanta**, 2015, 144, 340−355.	3.545	Q1 11/74 Chemistry, Analytical
Characterization of Stevia leaves by LC−QTOF MS/MS analysis of polar and non-polar extracts. **Food Chemistry**, 2017, 219, 329−338.	4.052	D1 7/125 Food Science & Technology
Development and application of a quantitative method besed on LC−QqQ MS/MS for determination of steviol glycosides in Stevia leaves. **Talanta**, 2016, 154, 263−269	4.035	Q1 9/75 Chemistry, Analytical

HS−GC/MS volatile profile of different varieties of garlic and their behavior under heating. **Analytical and Bioanalytical Chemistry**, 2016, 408, 3843−3852.	3.125	Q1 15/75 Chemistry, Analytical
Headspace−GC−MS volatile profile of black garlic vs fresh garlic: Evolution along fermentation and behavior under heating **LWT−Food Science and Technology**, 2017, 80, 98−105.	2.711	Q1 23/125 Food Science and Technology

AGRADECIMIENTOS

En primer lugar, quiero agradecer a mis directores de Tesis por su dedicación, su apoyo y su esfuerzo a lo largo de estos años. A la Catedrática María Dolores Luque de Castro, por abrirme las puertas el primer día y darme la oportunidad de realizar esta Tesis Doctoral bajo su dirección. Sus consejos, su experiencia y su sabiduría me han guiado durante estos años y me han permitido llegar hasta la meta que supone la presentación de esta Tesis Doctoral. Al Doctor Feliciano Priego Capote, por estar siempre a mi disposición y ser paciente a lo largo de mi aprendizaje. Su visión y enfoque brillante en la investigación ha influido en gran medida en que esta Tesis Doctoral llegara a buen puerto. A ellos les agradezco enormemente el haber confiado en mis capacidades, así como su dedicación y enseñanzas que me han permitido, en definitiva, llegar a ser la profesional que soy hoy.

En segundo lugar, quiero agradecer a todos mis compañeros en el grupo de investigación tanto su ayuda profesional como su compañerismo. A Mara y Miguel porque, aunque coincidí poco tiempo con ellos, me dieron una agradable bienvenida al grupo. A José, vecino de laboratorio, por ser una persona con una alegría infinita y tener siempre cosas nuevas e interesantes que contar. A Carlos, por ser uno de los primeros en guiarme en la investigación, siempre he tenido en cuenta todos sus consejos. A Pili, por esos momentos a la hora de comer donde contábamos nuestras anécdotas personales y esos ratitos de gimnasio que nos recargaban las pilas. A Vero, por derrochar tanta naturalidad y ser el alma de la fiesta en cualquier situación. A Ángela, por esos buenos ratos de chistes malos y presumiendo de sobrinas que recargaban el ánimo. A Fabiola, por haber sido encantadora como compañera y amiga durante su paso por España. A María del Mar y a Asun, por hacer más amenas las comidas y el día a día en el laboratorio.

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"La ciencia no es sino una perversión de sí misma, a menos que tenga como objeto final el mejoramiento de la humanidad"

Nikola Tesla

ÍNDICE

OBJETIVOS

El *objetivo genérico* de la Tesis fue contribuir al desarrollo de la metabolómica vegetal en tres ámbitos que se consideran de interés actual: (1) los residuos de la industria agroalimentaria mediante el estudio exhaustivo de su composición para proporcionar un mayor valor añadido a la industria en cuestión —la de zumos de cítricos, naranja en este caso. (2) Los nuevos cultivos que, aprovechando la diversidad de climas de nuestro país, se pueden importar para diversificar y enriquecer nuestra agricultura y de los cuales se requiere un adecuado conocimiento de la composición. La estevia rebaudiana se seleccionó como ejemplo representativo cuyo interés como edulcorante ya ha sido reconocido y atribuido a algunos glicósidos de esteviol, de los que es necesaria mayor información así como de los efectos de esta planta frente a enfermedades, que requieren justificación a través de la presencia y concentración de los metabolitos que los producen. (3) Los nuevos productos fruto de la manipulación industrial de cultivos tradicionales como el ajo. El proceso de fermentación del ajo fresco produce el llamado ajo negro, del que se requiere conocer su composición cuali y cuantitativa, así como llevar a cabo una comparación con el ajo fresco del que proviene para soportar fehacientemente la evolución a lo largo del proceso y las cualidades del producto final.

Tres han sido los pilares base de la investigación realizada: (i) el equipamiento analítico que posee el grupo en el que se integra la doctoranda, que incluye los dispositivos adecuados para la preparación de la muestra en cada caso, los instrumentos de última generación que se requieren para llevar a cabo los estudios metabolómicos, y los software y herramientas de análisis de datos

útiles para transformar la información analítica en información bioquímica. (ii) La existencia en el grupo de investigadores con la adecuada formación para planificar, guiar, atender, aclarar y discutir la investigación. (iii) La formación química de la doctoranda, su interés por la investigación, su disponibilidad para aceptar los retos planteados y su capacidad para solventarlos. En esta triple base se han soportado los *objetivos concretos*, que se han dividido en 3 partes a tenor de la materia abarcada:

Parte A:

- Un estudio bibliográfico, como primera toma de contacto con el campo en el que se desarrolla la investigación, ha dado lugar a la publicación de un capítulo en un libro multiautor y que, con el título "Extracción, separación e identificación/cuantificación de compuestos de interés en materiales agroalimentarios", pone de manifiesto que el objetivo de conocer la bibliografía existente se ha cumplido. Constituye éste el Capítulo I de la Tesis.
- Los estudios dedicados a la piel de naranja fueron la primera toma de contacto de la doctoranda con la investigación aplicada en los aspectos en los que el grupo tiene más experiencia: la preparación de la muestra, la identificación y la cuantificación de los componentes de la muestra analítica.

  La extracción simultánea de compuestos polares y apolares de la piel de naranja utilizando extractantes inmiscibles se consiguió con auxilio de ultrasonidos, que favorecen la formación de emulsión y la lixiviación. La identificación de los metabolitos en ambos extractos se realizó mediante LC–QTOF MS/MS y dio lugar al Capítulo II de la Tesis; mientras que la cuantificación de la familia de los flavonoides mediante un equipo LC–QqQ MS/MS junto con el estudio del pretratamiento de la muestra conforman el

Capítulo III.

Parte B:

- Los estudios, escasos e incompletos, que existen sobre la estevia rebaudiana y el interés creciente por las propiedades de esta planta impulsaron la investigación que se recoge en esta parte de la Tesis y que se centró primeramente en la caracterización de los metabolitos de esta planta en extractos obtenidos con medios polares y apolares y utilizando LC–QTOF MS/MS. Con este estudio se consiguió la identificación en grado de tentativa de 89 compuestos, de los cuales un buen número fueron identificados por primera vez, tal como recoge el Capítulo IV de esta Memoria.
- La cuantificación de compuestos de la estevia se llevó a cabo con un equipo LC–QqQ MS/MS que, en el caso de los glicósidos de esteviol, permitió alcanzar los límites de cuantificación más bajos conseguidos hasta la fecha de acuerdo con la bibliografía. Este estudio, recogido en el Capítulo V, permitió detectar las diferencias en los niveles de estos metabolitos en plantas cultivadas en campo frente a las de laboratorio e invernadero. En el caso de los compuestos fenólicos, muchos más numerosos en la estevia rebaudiana, se requirió un método dual para cuantificar los 33 que se detectaron en esta planta; método que se utilizó para evaluar la similitud entre variedades de la planta y correlacionar los niveles de estos metabolitos, tal como se muestra en el Capítulo VI de la Memoria.

Parte C:

- El ajo es un ingrediente básico de la cocina a escala mundial del que se han demostrado ampliamente sus propiedades beneficiosas para la salud. También el ajo negro como producto de la fermentación del ajo fresco ha

mostrado poseer cualidades beneficiosas para la salud, en algunos aspectos diferentes de las del fresco. Ambos tipos de ajo han sido insuficientemente estudiados (especialmente el negro) y ambos poseen componentes característicos volátiles y no volátiles. Por tanto, la investigación en esta área ha estado dirigida al estudio de la fracción volátil y de la polar en ajo fresco y en ajo negro, y a la evolución de ambas durante el proceso de fermentación.

- Para el estudio de la fracción volátil de ajo fresco se utilizó un equipo formado por un dispositivo de espacio de cabeza (HS) acoplado a un cromatógrafo de gases conectado a un espectrómetro de masas (HS–GC–MS). Con él se identificó el perfil de volátiles de las 3 variedades mayoritarias de ajo fresco; perfil que consta de 45 compuestos, de los cuales 17 se identificaron por primera vez, y que permitió establecer la variedad más rica en cada uno de los compuestos de interés, tal como se recoge en el Capítulo VII.
- El mismo equipo, HS–GC–MS, se utilizó para estudiar la composición de los componentes volátiles del ajo negro y la evolución en la formación de estos compuestos desde el ajo fresco hasta el producto final a lo largo del proceso de fermentación. Se identificaron en ajo negro un total de 51 compuestos volátiles, con cambios significativos respecto a este tipo de compuestos en ajo fresco, tal como muestra la comparación de sus perfiles. Se pone así de manifiesto que la fermentación da lugar a un gran número de transformaciones químicas, que pudieron seguirse a través del muestreo a tiempos prefijados y que se recogen en el Capítulo VIII de la Memoria.
- El estudio de la composición del ajo negro en comparación con la del fresco en cuanto a componentes polares se refiere se llevó a cabo mediante LC–QTOF MS/MS. Con este equipo se identificaron tentativamente 93 y 80 compuestos en ajo negro y fresco, respectivamente. El análisis estadístico permitió evaluar las diferencias globales de concentración entre las variedades de ajo fresco y negro, así como las existentes entre ambos tipos de

productos. Esta investigación se incluye en el Capítulo IX.

- Un último capítulo de la Tesis (Capítulo X) ha completado los objetivos concretos que se persiguieron al planificar la Tesis. Para este estudio también se utilizó el equipo LC–QTOF MS/MS del capítulo anterior, con el que se realizó un seguimiento de la evolución que experimentan los compuestos polares del ajo a medida que avanza el proceso de fermentación. Los tiempos a los que se producen los cambios más significativos y las posibles vías metabólicas a través de las cuales tienen lugar son de enorme interés para explicar la composición final del ajo negro.

Otros aspectos que han contribuido a que la futura doctora adquiera una amplia formación, *objetivo último* de toda Tesis doctoral, han sido: (i) un capítulo de libro multiautor, que pone de manifiesto su formación en el área de la metabolómica, en este caso aplicada al estudio de los carotenoides (Anexo I); (ii) comunicaciones orales en 3 conferencias nacionales (Anexo II); y (iii) otras actividades formativas realizadas por la doctoranda (Anexo III).

INTRODUCCIÓN

El desarrollo de la investigación que ha hecho posible esta Tesis Doctoral ha conllevado la formación de la doctoranda en un número de aspectos analíticos y metabolómicos de los cuales se ofrece una panorámica en esta introducción. Se pretende así poner de manifiesto la formación adquirida por la doctoranda como base de la labor investigadora que ha desarrollado. En algún caso se incluirán de forma sucinta los resultados de su investigación para subrayar que ésta ha servido para incluir un eslabón más en una determinada cadena de logros anteriores.

Focalizadas siempre en el área agroalimentaria, se considerarán las formas de muestreo y de conservación de las muestras, de su preparación y su análisis para terminar con el fin último de toda la cadena analítica desarrollada: obtener información metabolómica sobre la muestra en cuestión o sobre una parte de ella representativa del sistema en estudio. Por tanto, esta introducción versará sobre: (1) Los tipos de muestras vegetales comunes en el área agroalimentaria. (2) Las formas de recogida y conservación de las muestras vegetales, que serán función de su naturaleza y de la información que se pretenda conseguir de ellas. (3) La preparación de la muestra, con las ventajas e inconvenientes que plantea el uso de procedimientos tradicionales o nuevas vías que pretenden conseguir una mayor velocidad y/o automatización del procedimiento, mayor eficiencia, menor contaminación, etc. (4) Las técnicas de detección, acopladas o no a un equipo de alta resolución en la separación de los componentes. (5) El tratamiento de los datos, cuya complejidad será función de la capacidad del detector para proporcionarlos. (6) La metabolómica como disciplina que, a través de un uso adecuado de las anteriores herramientas analíticas, proporcionará la información perseguida sobre

los compuestos objeto del estudio y sobre las rutas metabólicas en las que están implicados.

1. Tipos de muestras agroalimentarias

Dentro del área de la agroalimentación existen varios tipos de muestras que son objeto de estudio en función de la información que se pretende obtener de ellas. El primer tipo la componen las plantas o partes de plantas (por ejemplo, las hojas o los frutos) de las cuales se pretende estudiar una propiedad de interés para un buen desarrollo de la misma o para la alimentación y la salud del ser humano. A este tipo de planta pertenece la estevia rebaudiana, seleccionada para los estudios recogidos en la parte B de esta Memoria. Esta planta se está popularizando actualmente debido al poder edulcorante que presentan sus hojas, por lo que en estos estudios se pretende caracterizar los compuestos presentes en las hojas, así como cuantificar dos familias representativas de esta planta como los glicósidos de esteviol y los compuestos fenólicos. Si bien se han hecho algunos estudios sobre esta planta dirigidos sobre todo a la extracción de los principales glicósidos de esteviol, poco se sabe del resto de compuestos que están presentes en las hojas. Como ejemplo, únicamente Karakose *et al.* estudiaron con anterioridad el perfil fenólico de las hojas de estevia rebaudiana [1,2]. El segundo tipo de muestras lo forman los productos derivados de vegetales, principalmente alimentos procesados, ya sea física o químicamente. Las investigaciones llevadas a cabo con este tipo de muestras se centran principalmente en los cambios producidos en el alimento durante el proceso, que pueden alterar la composición de la materia prima de forma positiva o negativa. De este tipo de muestras se seleccionó el ajo fermentado o ajo negro, que dio lugar a los estudios recogidos en la parte C de esta Memoria, donde se compara su composición con la del ajo fresco, así como la evolución que experimenta durante la fermentación. Ejemplo de la experiencia del grupo en el que se integra la doctoranda en este tipo de estudios

es la comparación de los perfiles de los compuestos fenólicos en aceite de oliva virgen procedente de distintas variedades de aceituna [3].

Por último, el tercer tipo de estudios se orientó a la valorización de residuos de la industria agroalimentaria generados en el procesado. Existen numerosas industrias, tales como las de producción de aceites, vinos o zumos, que generan una gran cantidad de desechos vegetales, principalmente formados por el hueso y/o la piel cuando el proceso implica un determinado fruto. Estos desechos generalmente no tienen uso o se destinan a alimento de ganado; sin embargo, se ha demostrado que en muchos casos son ricos en compuestos de gran interés para el ser humano. Por tanto, el fin último de los estudios realizados con estas muestras es su explotación para obtener unos compuestos utilizables a la vez que se disminuye la cantidad de residuos generados en estas industrias. En esta Memoria, los trabajos recogidos en la parte A se han orientado a la caracterización de la piel de naranja, principal desecho de la industria de este tipo zumos, así como a la demostración de su interés como fuente importante de flavonoides. Como ejemplo de trabajos en este ámbito realizados en el grupo de investigación en el que se integra la doctoranda está la caracterizaron la fracción polar y medio polar de las lías del vino, el principal residuo generado en la industria vinícola. En este estudio se identificaron una gran cantidad de compuestos fenólicos, así como aminoácidos y antocianinas, poniendo de manifiesto su alto potencial como fuente de estos compuestos [4].

2. Recogida, conservación y pretratamiento de la muestra vegetal

Las características genéricas de las muestras vegetales hacen que tanto su recogida como su conservación tengan unos aspectos que las distinguen de otras muestras y que varían aun dentro de ellas en función de sus características específicas y del objetivo que se pretenda tras el muestreo y la conservación.

2.1. La recogida de la muestra vegetal

La información que se pretende obtener de la muestra establece la forma de muestreo, que puede ser muy variada. Siempre que sea posible —y para conseguir una característica analítica de enorme importancia como es la representatividad— el muestreo debe abarcar varias unidades del sistema en estudio (e.g., plantas, árboles). Además, existen numerosas variables que influyen el estado de la muestra y que hay que tener en cuenta a la hora de muestrear. Una de ellas es el momento del día en el que se recoge la muestra, ya que se ha demostrado que la concentración de los metabolitos en la planta cambia a lo largo del día. Por ejemplo, Urbanczyk-Wochniak *et al.* estudiaron los metabolitos presentes en la hoja de la patata (*Solanum tuberosum*) en diferentes momentos del día, comprobando que 56 metabolitos cambiaban significativamente su concentración a lo largo del día [5].

Otra variable clave es la edad de la planta, así como el punto de maduración de las hojas o el fruto. En este caso si lo que se pretende es realizar el seguimiento a lo largo del crecimiento o la maduración, la complejidad del diseño de muestreo dependerá del rigor requerido. Un ejemplo de diseño de muestreo para el estudio de la maduración del fruto es el realizado por Ledesma Escobar en el caso del limón persa (*Citrus latifolia*), que se prolongó durante el tiempo requerido para la maduración: 14 semanas [6]. Consistió en un muestreo por coordenadas rectangulares considerando la superficie de la parcela como un rectángulo, tal como se muestra en la Fig. 1.a. En la primera semana se etiquetaron las ramas que presentaban floración y estaban más próximas a cada coordenada aleatoria. La recolección de los frutos se realizó en las semanas 1, 3, 5, 7, 9, 12 y 14 de maduración de los frutos. Parte de los frutos maduros se conservaron a 25 ^{o}C durante 2 semanas, con lo que el muestreo total es el recogido en la Fig. 1.b.

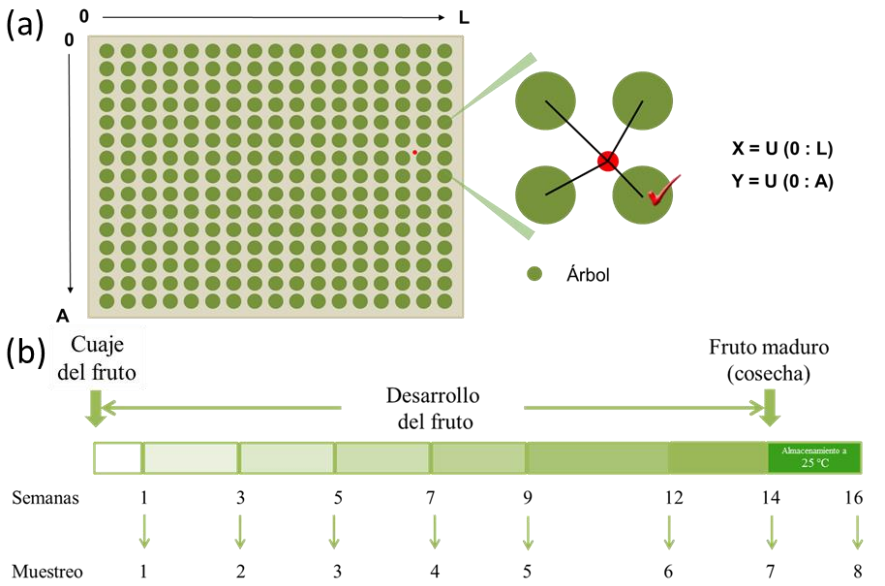

Figura 1. Esquema general del muestreo por coordenadas aleatorias. El gráfico de la derecha muestra el criterio de selección de las muestras, mientras que la tabla representa el plan general de muestreo del limón persa. Con permiso de Ledesma Escobar.

Otro aspecto a tener en cuenta es la parte de la planta que se utiliza para el estudio debido a que el contenido en metabolitos puede variar drásticamente entre hojas, tronco, raíces, flores o frutos. El estudio recogido en el Capítulo V de esta Memoria aborda este aspecto ya que en él se comparan las hojas y las hojas más las ramas de la planta estevia rebaudiana como muestra para la extracción y cuantificación de glicósidos de eteviol.

2.2. La conservación y el pretratamiento de la muestra vegetal

También la complejidad de esta etapa, como la de cualquier etapa analítica, depende del objetivo final del análisis. La muestra fresca, recién obtenida, es difícil de mantener hasta el comienzo de su preparación, por lo que generalmente es necesario algún tipo de pretratamiento para su conservación, que puede consistir en congelación en frigorífico, congelación/molturación en atmósfera de nitrógeno

líquido, deshidratación en corriente de aire, secado en horno o liofilización, entre las más comunes. La eliminación de agua de la muestra es un paso crucial ya que la mayor parte de las reacciones químicas de las plantas se llevan a cabo en medio acuoso. Se evita de esta forma que se produzcan reacciones de degradación u oxidación de los metabolitos. Además, este paso afecta a la detección de los metabolitos en algunos casos, especialmente cuando se utiliza la Resonancia Magnética Nuclear (NMR) ya que el agua es un potente interferente en la detección de los metabolitos con esta técnica [7].

En todos los casos es necesario asegurar la estabilidad de los compuestos de interés en las condiciones de conservación (*e.g.*, estabilidad térmica si se realiza el secado en horno, estabilidad oxidativa si se realiza en corriente de aire) o comparar varias de estas formas para conocer cuál se adecua más al caso en estudio [8]. Un estudio reciente sobre la estabilidad de los componentes polares del limón en las condiciones de conservación (secado en corriente de aire y liofilización) en comparación con la muestra fresca puso de manifiesto que: (i) de los 74 metabolitos tentativamente identificados 4 no se encontraron en las muestras liofilizadas. (ii) La concentración relativa de 44 de los metabolitos comunes difería de forma significativa dependiendo de la forma de conservación. (iii) La diferencia en concentración se atribuyó al efecto de la liofilización o del secado en corriente de aire sobre las correspondientes rutas metabólicas y no sólo a la termolabilidad de los metabolitos [9].

La aportación realizada en esta Tesis sobre este aspecto concreto del proceso analítico se orientó a la cuantificación de flavonoides mediante LC–QqQ en piel de naranja liofilizada y secada en horno en comparación con la piel fresca, utilizando 8 variedades de naranja. Los resultados, recogidos en el Capítulo III, muestran que la liofilización preserva la concentración de los metabolitos en estudio, mientras que el secado en horno disminuye la concentración de las formas glicosiladas en beneficio de las agliconas. Por tanto, este estudio marca la pauta a

seguir en la conservación de la materia prima en función de que el producto de interés sea una u otra de las formas.

Por último, el pretratamiento de la muestra tiene como objetivo la eliminación de partículas en suspensión en el caso de muestras líquida y la homogeneización y la disminución del tamaño de partícula en el caso de muestras sólidas. Esto es especialmente necesario cuando se pretende realizar la extracción de los metabolitos presentes en la muestra. La homogeneización de la muestra permite que las porciones seleccionadas en cualquier momento sean iguales, con lo que se alcanza una mayor reproducibilidad en la determinación de metabolitos. Además, la disminución del tamaño de partícula permite un mayor contacto entre la muestra y el extractante, haciendo que la extracción sea más efectiva. El efecto de esta variable se puso de manifiesto en el estudio de Chupin *et al*., en el que se extrajeron compuestos de interés del pino marítimo (*Pinus Pinaster*) usando muestras con distinto tamaño de partícula. Se consiguió una mejora significativa en la extracción usando un tamaño de partícula de las muestras inferior a 400 μm [10].

3. Preparación de la muestra vegetal

La preparación de la muestra es la etapa cuyo resultado es la obtención de la muestra analítica, es decir, aquella porción de la muestra inicial que contiene los analitos en la forma más adecuada para su introducción en el detector o en un equipo de separación con alto poder de resolución como etapa previa a la detección [11]. Generalmente sólo una parte de los componentes de la muestra inicial son de interés en cada estudio concreto: los compuestos polares, los no polares, los volátiles, etc.

Cuando los compuestos de interés son volátiles (aromas, generalmente) se puede conseguir su separación selectiva mediante hidrodestilación [12],

destilación por arrastre con vapor [13], o mediante espacio de cabeza, tal como se ha realizado en el estudio de los compuestos volátiles del ajo que se recoge en los Capítulos VII y VIII de la Memoria. La técnica llamada de espacio de cabeza recibe el nombre de la zona que se utiliza en el contenedor de la muestra para conseguir la parte de ella que es volátil y que ocupa, tras el calentamiento adecuado, la parte superior del contenedor no ocupada por la muestra. El muestreo de este espacio puede ser estático [14] o dinámico [15]—en este último caso los compuestos de la muestra que se han evaporado al espacio de cabeza se arrastran por un gas hasta un sorbente para su recogida durante un tiempo pre-establecido [16].

Para muestras líquidas se utiliza generalmente la extracción líquido−líquido, una técnica simple que se basa en poner en contacto la muestra con un solvente inmiscible donde los metabolitos de interés sean más solubles que en la muestra original [17]. Otra alternativa es el uso de un sólido como sorbente que retiene los compuestos de interés, dando lugar a la extracción en fase sólida, en formato normal (SPE) o en formato micro (SPME) [18]. La SPME también se usa para la extracción y preconcentración de compuestos volátiles procedentes del espacio de cabeza. Como ejemplo, Farag *et al.* determinaron los volátiles procedentes del hibisco (*Hibiscus sabdariffa*) usando la SPME del espacio de cabeza generado al calentar las flores a 50 ºC durante 30 min [19].

La forma más común de obtener los compuestos polares o los no polares de una muestra sólida, vegetal o no, es una etapa de extracción sólido–líquido (más precisamente, una etapa de lixiviación) utilizando un extractante con afinidad por las especies de interés. En mucha menor proporción se utiliza la dispersión de la matriz en una fase sólida (matrix solid-phase dispersion —MSPD) [20]. Esta técnica tiene como principal inconveniente la lentitud, ya que el paso de la muestra en suspensión a través de la columna se realiza por gravedad. La digestión de la muestra vegetal se lleva a cabo en muy contadas ocasiones, ya que su disolución total, junto con los reactivos utilizados para obtener el digerido, da lugar a una

muestra analítica muy compleja.

La lixiviación es la técnica más ampliamente utilizada para separar los compuestos de interés de la matriz vegetal sólida. Esta separación será tanto más selectiva y eficaz cuanto mayor afinidad tengan estos compuestos por el lixiviante y este menos por el resto de los componentes de la muestra. Los métodos tradicionales de lixiviación están basados en maceración o en el uso de un extractor soxhlet; ambos requieren un equipamiento simple, pero los tiempos requeridos para esta etapa pueden ser largos y los volúmenes de lixiviantes grandes en comparación con otras técnicas más actuales [21]. Sin embargo, la maceración se usa ampliamente en los laboratorios de investigación debido a que el ahorro en extractante respecto al que se requiere en las técnicas más recientes es mínimo, mientras que la simplicidad de esta técnica permite realizar varias extracciones al mismo tiempo con una instrumentación mínima, reduciendo así el tiempo requerido para llevar a cabo todas las extracciones necesarias en cada estudio.

Existen, además, numerosos compuestos en matrices vegetales que son fácilmente extraíbles y no requieren energía adicional para favorecer la transferencia de los mismos al extractante. Esto ocurre con las aflatoxinas, compuestos que están presentes en ciertas matrices vegetales y que son tóxicas. Se ha demostrado que estos compuestos son fácilmente extraíbles usando extractantes de polaridad media sin la aplicación de energía que favorezca su extracción; sin embargo, se han desarrollado numerosas metodologías, a veces complejas, para su extracción [22]. El uso de estas metodologías implica un consumo innecesario de energía, recursos y tiempo, por lo que es importante una buena elección de la técnica de extracción en función de la naturaleza de los compuestos de interés. Algo semejante ocurre con los glicósidos de esteviol, ya que en la literatura hay numerosos casos en los que se proponen metodologías complejas y dispares para su extracción [23–35].

En el Capítulo V de esta Memoria se lleva a cabo una extracción completa de

estos compuestos mediante un método simple basado en la maceración a temperatura ambiente. Se pone así de manifiesto que, aunque en la última década se ha promovido el desarrollo de alternativas para eliminar o minimizar los aspectos negativos que presentan la maceración o la extracción con soxhlet (*e.g.*, uso de líquidos sobrecalentados, en estado supercrítico, o la asistencia de energías auxiliares) hay que considerar críticamente si se requiere la utilización de tales alternativas.

3.1. Lixiviación con líquidos sobrecalentados o en estado supercrítico

Los líquidos sobrecalentados (líquidos calentados por encima de su punto de ebullición, pero sometidos a presión suficiente para mantener el estado líquido) proporcionan una gran eficiencia en la lixiviación, que se realiza en tiempos muy cortos y con volúmenes de lixiviantes muy pequeños. La lixiviación con líquidos sobrecalentados (SHLE) es versátil, ya que puede desarrollarse en régimen estático, dinámico o en una combinación de ambos [36–38], con equipos diseñados en el laboratorio o comerciales. Como ejemplo, Shabkhiz *et al.* emplearon la extracción con agua sobrecalentada en régimen dinámico para obtener el ácido glicirrícico a partir de la raíz de la planta regaliz (*Glycyrrhiza glabra*). En este estudio también se comparó esta técnica con la extracción con soxhlet y con ultrasonidos, obteniendo una mayor cantidad de ácido glicirrícico con la extracción con agua sobrecalentada [39].

Un fluido supercrítico es aquel que se encuentra a temperatura y presión por encima de su punto crítico. Sus propiedades exageradamente ensalzadas en la pasada década de los 90 —especialmente en el caso del CO_2 supercrítico— los hicieron parecer los lixiviantes y las fases móviles cromatográficas ideales. Los problemas derivados del diferente comportamiento de las muestras fortalecidas con los analitos con respecto a aquéllas que los contenían de forma natural condujo al estado actual de la extracción con fluidos supercríticos (SFE), prácticamente limitada al uso de la extracción de grasas en alimentos [40] o de compuestos no

polares de alto valor añadido, tanto a escala de laboratorio como industrial [41].

3.2. Lixiviación asistida por microondas o por ultrasonidos

En las condiciones óptimas de aplicación, el auxilio de una fuente externa de energía, tal como microondas (MW) o ultrasonidos (US) puede mejorar drásticamente le etapa de lixiviación, tanto en eficacia como en tiempo para su desarrollo y en volumen de lixiviante necesario [42]; incluso posibilitar su automatización.

La extracción asistida por MW (MAE) puede emplearse de diferentes formas dependiendo del modo de aplicación de las MW (que pueden ser focalizadas o no), continua o discontinua, en sistemas abiertos o cerrados [43]. Además, se han desarrollado nuevas alternativas en la MAE para la extracción de compuestos en plantas, como son la extracción en atmósfera de nitrógeno [44] o en vacío [45]; con el uso combinado de MW y US [46], o incluso en ausencia de extractante [47]. Esta variedad de modalidades permite elegir la forma de trabajo más adecuada para cada caso en estudio dando lugar a métodos MAE rápidos y efectivos que ponen de manifiesto la versatilidad de la técnica. Es destacable el hecho de que la aplicación de MW destruye la matriz vegetal de la muestra por rotura de la pared celular, favoreciendo así la extracción de los metabolitos. Publicaciones recientes que comparan la MAE con otras técnicas de extracción ponen de manifiesto su eficacia para la extracción de ciertos compuestos en matrices vegetales. Un ejemplo de este comportamiento es el estudio de Xie *et al.*, en el que comparó la MAE con la extracción asistida por US (USAE) y con la extracción con soxhlet para la obtención de alcaloides de la planta *Stephania sínica*. La MAE necesitó un tiempo de extracción de 1.5 min, mientras que la USAE y la extracción con soxhlet requirieron 30 y 360 min, respectivamente [48]. Sin embargo, la eficacia de la MAE depende estrechamente de la naturaleza del extractante —deseablemente polar— y no es recomendable para la extracción de compuestos termolábiles ya que la aplicación de MW aumenta la temperatura del sistema muestra−extractante.

Por otro lado, la USAE tiene las ventajas de la ausencia de restricciones en cuanto a la polaridad del extractante y del escaso incremento de temperatura que se produce en el medio por aplicación de este tipo de energía. Además, los US producen el efecto de cavitación que promueve la formación de emulsiones de solventes inmiscibles, lo que favorece la extracción de compuestos de distinta polaridad en una sola etapa [49]. Esta técnica fue la seleccionada para la extracción de metabolitos polares y no polares de la piel de naranja en el estudio que se recoge en el Capítulo II. Algunos métodos basados en la USAE emplean como fuentes de esta energía baños de US de los utilizados para la limpieza de material de laboratorio y para la desgasificación de disoluciones –especialmente de fases móviles cromatográficas. Estos dispositivos son poco reproducibles ya que la distribución de la energía de US en ellos no es uniforme y experimentan el fenómeno de fatiga o pérdida de potencia con el tiempo. Mucho más eficaces son las sondas o los reactores de US, que tienen un comportamiento constante con el tiempo de aplicación y permiten la optimización de parámetros como la amplitud y/o el ciclo útil [50]. Un ejemplo del empleo de la USAE es el método desarrollado por Da Porto *et al.* para la extracción de aceite de las semillas de la uva (*Vitis vinífera* L.), que fue comparado con la extracción soxhlet. Para obtener el mismo rendimiento, el método de USAE requirió 30 min, mientras que el método soxhlet 6 h, poniendo de manifiesto que la USAE acelera la extracción de aceites. Dentro del mismo estudio Da Porto *et al.* también desarrolló un método de USAE para la extracción de fenoles y lo comparó con la maceración. En este caso, la USAE necesitó 15 min para obtener la mayor cantidad de fenoles, mientras que la extracción con maceración se llevó a cabo durante 12 h aunque sin estudiar tiempos de extracción menores. Sin embargo, la USAE no conllevó mejoras significativas en el rendimiento de extracción de fenoles, siendo éste un claro ejemplo de compuestos fácilmente extraíbles que no requieren energías auxiliares para aumentar el rendimiento [51].

Una última etapa que se requiere en muchos casos para la obtención de una muestra analítica adecuada para su introducción en el equipo de separación de alto poder de resolución es la de limpieza con el fin de eliminar partículas en suspensión presentes en el extracto. La filtración o la centrifugación son las técnicas más simples, aunque existen alternativas como la extracción sólido−líquido dispersiva que se utiliza no sólo como técnica de limpieza sino también de preconcentración de los compuestos a determinar. Por ejemplo, Demes *et al.* emplearon esta técnica para la limpieza y concentración previas a la determinación de residuos de pesticidas a nivel traza en aceites comestibles de girasol, salvado de arroz y cacahuete [52].

También en algunos casos es necesaria una etapa de derivatización previa al análisis. La etapa consiste en una reacción química en la que se le agrega un grupo funcional o se enlaza un compuesto a los metabolitos a determinar con el fin de facilitar su separación individual y/o su detección. Ejemplos de esta etapa son la formación de compuestos fluorescentes utilizando diferentes agentes derivatizantes [53,54], o la adición de grupos funcionales que aumentan la volatilidad de los metabolitos para facilitar su separación mediante cromatografía de gases [55]. Un ejemplo muy común de este último tipo de derivatización es la metilación de los ácidos grasos para su separación mediante cromatografía de gases y posterior determinación mediante un detector de ionización en llama o un espectrómetro de masas. En el grupo en el que se integra la doctoranda se ha utilizado la metilación en diferentes casos, como en la determinación de ácidos grasos esterificados y no esterificados en el aceite de oliva virgen obtenidos de diferentes variedades de aceituna [56] y en la aceituna durante su crecimiento [57].

4. Análisis del extracto vegetal

Los tipos de análisis comúnmente empleados para la obtención de información a partir de muestras vegetales pueden clasificarse en tres grupos principales en función de la instrumentación utilizada y de la información obtenida: (i) análisis de una propiedad determinada; (ii) análisis directo del extracto o incluso de la muestra sin una etapa previa de extracción; y, (iii) análisis con separación previa.

4.1. Análisis de una propiedad determinada

El material vegetal generalmente es rico en compuestos a los que se le atribuyen propiedades específicas, muchas de ellas beneficiosas para el organismo, como por ejemplo capacidad antioxidante o capacidad de eliminar radicales libres. Existen diferentes metodologías de análisis de estas propiedades que están bien establecidas y son ampliamente usadas en el análisis de muestras vegetales. Son metodologías simples y rápidas, basadas principalmente en cambios en la absorbancia del extracto por la adición de un reactivo específico, que presentan una baja selectividad ya que son varias las familias de compuestos que contribuyen a la señal monitorizada; por lo que no se puede obtener información individual de los compuestos presentes en el extracto [58]. Se requiere en todo caso realizar una recta de calibrado —utilizando un único patrón genérico para todos los compuestos— que relacione la absorbancia con la concentración de los compuestos.

Algunas de estas metodologías proporcionan el contenido total en la muestra de una familia de compuestos concreta, como por ejemplo fenoles o antocianinas. El índice total de fenoles (TPI) y el contenido total de antocianinas (TAC) relacionan la cantidad de estos compuestos con la absorbancia de la solución a 280 nm y 520 nm, respectivamente. El método de Folin-Ciocalteu (F−C) es el más empleado para determinar la cantidad total de antioxidantes fenólicos. En él se

utiliza el reactivo F−C (disolución de fosfomolibdato y fosfowolframato) que se reduce en presencia de antioxidantes formando un compuesto azulado que absorbe a 765 nm [59]. La reducción de ion férrico complejado también se usa ampliamente para la determinación de la capacidad antioxidante (ensayo FRAP). En él, un complejo de hierro (III) se reduce a hierro (II) en presencia de antioxidantes en medio ácido. El complejo reducido se caracteriza por un color azul que presenta un máximo de absorbancia a 593 nm. Otro de los métodos frecuentemente utilizado es la medida de la capacidad de eliminación de radicales libres usando 2,2-difenil-1-picrilhidrazilo (DPPH). Este compuesto actúa como un radical libre estable con un intenso color violeta que cambia a incoloro o amarillo pálido cuando se neutraliza; por lo que en este caso lo que se mide es la pérdida de absorbancia a 520 nm [60].

Un ejemplo del uso de estas metodologías es el trabajo de Moo-Huchin *et al.*, que determinaron la cantidad total de fenoles, flavonoides, antocianinas y carotenoides, así como la capacidad antioxidante y de eliminación de radicales libres en la piel de la fruta del caimito (*Chrysophyllum cainito* L.) y del anacardo amarillo y rojo (*Anacardium accidentale*) [61].

4.2. Análisis sin etapa de separación: análisis directo

Los análisis sin separación previa no proporcionan información de los compuestos individuales que constituyen la muestra analítica, aunque sí son útiles para obtener información global de la misma y en estudios comparativos entre diferentes tipos de muestras vegetales. Para estos análisis se usan principalmente NMR y espectrometría de masas (MS) y, en menor proporción, las espectroscopías infrarroja y Raman. La NMR es una técnica no destructiva, por tanto, muy útil para muestras valiosas, con una preparación de muestra simple y rápida (normalmente sólo requiere dilución). Además, permite obtener espectros NMR directamente de células y tejidos, así como monitorizar metabolitos sensibles al proceso de extracción. No obstante, presenta una baja sensibilidad comparada con MS,

requiere el uso de disolventes deuterados, principalmente metanol o cloroformo, y el agua presente en los sistemas biológicos ocasiona interferencia, por lo que debe llevarse a cabo la supresión de su señal. Esta técnica se basa en la absorción de energía de una frecuencia determinada por algunos núcleos atómicos sometidos a un campo magnético externo. Los núcleos atómicos presentes en compuestos biológicos capaces absorber esta energía son ^{1}H, ^{13}C, ^{15}N y ^{31}P, de los que generalmente se usan los dos primeros, dando lugar a las modalidades ^{1}H-NMR y ^{13}C-NMR. La absorción de cada uno de los núcleos depende de otros núcleos presentes en su entorno, por lo que es útil a la hora de determinar la estructura del metabolito en estudio. La NMR permite la combinación de diferentes espectros de una misma muestra —denominadas técnicas NMR bidimensionales— con lo que se puede conocer la interacción de los núcleos de una misma molécula [62]. Aunque estas técnicas proporcionan mayor información cualitativa que la MS, los tiempos de adquisición de datos son más largos, pudiendo ser de hasta un día. Como ejemplo del uso de la NMR para el análisis de muestras vegetales, De Falco *et al.* emplearon esta técnica para obtener el perfil composicional de los extractos polares y no polares de alcachofa globo (*Cynara cardunculus* L. var. *scolymus* L. Fiori) y de cardo (*Cynara cardunculus* L. var. *altillis* DC). Mediante el tratamiento estadístico de los datos obtenidos pudieron diferenciar entre diferentes variedades locales de Italia, tal y como se muestra en la Fig. 2 [63].

Las espectrometrías infrarroja y Raman también se han utilizada para la obtención del perfil metabolómico, ya que tienen un pequeño coste por análisis y tiempos de toma y tratamiento de datos cortos. Sin embargo, al contrario que la NMR, la información estructural que proporcionan es más limitada y son poco sensibles. Una ventaja importante que presenta el uso de la espectroscopía Raman es que pueden realizarse análisis *in vivo*, tal y como expone el trabajo de Rys *et al.*, en el que se usó esta técnica para evaluar el efecto de la sequía en las hojas de la

soja [64].

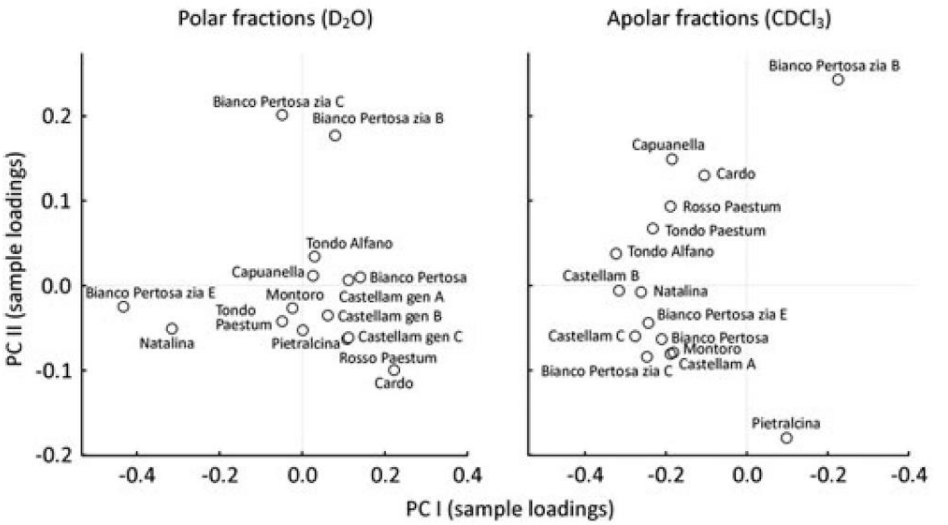

Figura 2. Diferenciación entre distintas variedades locales de alcachofa globo procedentes de Italia mediante su análisis por NMR sin separación previa. Con permiso de John Wiley and Sons.

Por otro lado, la MS es una técnica ampliamente utilizada en el estudio de muestras vegetales. En el análisis sin separación previa esta técnica presenta la ventaja respecto a otras de que un espectrómetro de masas se comporta, en cierto modo, como un equipo de separación ya que permite separar las señales en función de la relación masa/carga (*m/z*) de los compuestos [65]. Aunque la MS sin separación previa es una técnica muy útil para la obtención de un perfil composicional global, la combinación de MS y técnicas de separación previa ha ganado una gran importancia en el análisis de muestras vegetales, por lo que se explica más detalladamente en la subsección siguiente.

4.3. Análisis con etapa de separación previa

La introducción de una etapa de separación previa a la detección de los metabolitos es de gran importancia cuando se pretende cuantificar compuestos individuales o identificar los que forman parte de la muestra. Aunque existen otras

técnicas, como por ejemplo la electroforesis capilar, la cromatografía es la más empleada, donde los compuestos se distribuyen entre una fase móvil (un líquido o un gas) y una estacionaria (el relleno de la columna cromatográfica). La separación se produce en función de la afinidad de los compuestos hacia cada fase. En función de que se utilice un líquido o un gas como fase móvil, la técnica se denomina cromatografía líquida (LC) o gaseosa (GC).

La GC permite la separación de compuestos volátiles como, por ejemplo, compuestos aromáticos. Para la separación de compuestos con una menor volatilidad es necesario una etapa previa de derivatización, como se comentó en el apartado 3 de esta introducción. Esta técnica de separación es más limitada en comparación con la LC por el tipo de compuesto que permite separar, por lo que principalmente se ha usado para el análisis de compuestos volátiles [66], ácidos grasos previa derivatización [67] y pesticidas [68] en muestras vegetales.

Por otro lado, la LC posibilita la separación de un mayor número de compuestos y aporta una mayor versatilidad al análisis de muestras vegetales. Este comportamiento se debe principalmente a la existencia de diferentes formatos de cromatógrafos y la capacidad de selección tanto de columnas como de fases móviles idóneas en función de la naturaleza de los compuestos de interés. Existe una amplia gama de cromatógrafos que va desde los convencionales hasta los de ultra-resolución que, progresivamente, han disminuido algunos de los principales factores cromatográficos, como el diámetro interno y la longitud de la columna, el tamaño de partícula de relleno y el flujo de la fase móvil aumentando considerablemente la capacidad de separación de los cromatógrafos (ver Tabla 1). La nanoLC ha ganado mucho protagonismo en los últimos años debido a las ventajas que presenta frente a la HPLC convencional ya que, al utilizar columnas capilares de diámetro interno muy pequeño, disminuye considerablemente el consumo de fases móviles (los flujos oscilan entre 100 y 1000 nL/min) [69]. Se consigue así disminuir los volúmenes de inyección de muestra, con la consiguiente

reducción de costes y los beneficios medioambientales derivados. Se ha comprobado que una reducción en el diámetro interno de la columna aumenta la sensibilidad debido a que se produce una menor dilución del analito [70] y se consigue una mayor eficacia [71]. Además, la existencia de diferentes tipos de rellenos de columna permite elegir el más idóneo para la separación de los compuestos. Así, las columnas de fase reversa con un relleno apolar, generalmente sílice empaquetada (C8 o C18), presentan una fuerte interacción con compuestos de baja y media polaridad, mientras que las de fase normal con un relleno polar, como son las HILIC, son más eficaces para la separación de compuestos polares.

Tabla 1. Principales diferencias entre los distintos tipos de LC.

	UPLC/RRLC*	HPLC	MicroLC	LC capilar	NanoLC
Diámetro interno de la columna (mm)	1.5−4.5	1.5−4.5	0.8	0.18−0.32	0.075−0.1
Longitud de la columna (cm)	3−15	3−30	5−25	5−25	5−15
Tamaño de partícula (μm)	<2	3−40	3−5	3−5	3−5
Flujo de fase móvil (μL/min)	200−5000	200−5000	10−100	1−10	0.1−1

*Ultra performance liquid chromatography/Rapid resolution liquid chromatography

4.3.1. Herramientas analíticas para la detección con separación previa

Existen varios detectores acoplables a cromatógrafos para el análisis de muestras vegetales, pero la selección del detector idóneo depende principalmente de la cantidad de información que se pretenda obtener de la muestra. En primer lugar, existen detectores simples, como son el detector de diodos en fila (DAD) [72] o el de ionización en ionización en llama (FID) [73], que se usan generalmente en análisis sencillos de un número reducido de compuestos o en etapas de optimización de un método en las que la evolución global en función del cambio de las variables en estudio puede seguirse a través de la comparación del perfil

cromatográfico. Esos detectores son asequibles, fáciles de usar y de mantener, aunque la información que proporcionan es limitada.

También los detectores de NMR se ha usado recientemente acoplados a cromatógrafos para el análisis de muestras vegetales. Como ejemplo, Exarchou *et al.* emplearon este equipamiento (LC–NMR) para el estudio de los componentes mayoritarios de la planta *Salvia fruticosa* Mill. [74]. Sin embargo, la herramienta analítica de detección más frecuentemente usada para el estudio de muestras vegetales es la MS ya que presenta una mayor sensibilidad y mayor resolución cuando se analizan matrices complejas. Esta técnica de detección fue la seleccionada para los estudios realizados en esta Tesis Doctoral; por lo que se tratará a continuación en más profundidad.

La espectrometría de masas La MS se fundamenta en la ionización, posterior fragmentación y detección de los iones formados por los analitos presentes en la muestra, todo ello encadenado mediante el desplazamiento diferencial de las moléculas ionizadas (o átomos ionizados) por aplicación de un campo eléctrico en condiciones de vacío. En resumen, un espectrómetro de masas consiste en una fuente de iones, un analizador de masas, un detector y un sistema de recogida de datos. Las moléculas de la muestra analítica se insertan en la fuente de iones, donde se ionizan. Los iones así formados se encuentran en fase gaseosa y se separan entre sí en función de su relación masa/carga *(m/z)* en el analizador de masas, donde se detectan.

Existen diferentes vías de ionización para generar los iones dando lugar a diferentes fuentes de ionización. La ionización por impacto electrónico (EI) y la ionización química (CI) son las que se utilizan generalmente en el acoplamiento GC–MS, mientras que la ionización por electroespray (ESI) y la ionización química a presión atmosférica (APCI) son las alternativas más comunes en LC–MS [75]. Para la investigación recogida en esta Memoria se utilizó la ESI en los trabajos donde se empleó LC−MS y la EI en GC−MS.

La ESI es particularmente útil en el acoplamiento de MS con LC, ya es especialmente adecuada para compuestos polares e iónicos. Mediante esta fuente de ionización, la muestra se pulveriza en un metal o en un capilar de sílice fundida, dando lugar a un espray formado por gotitas cargadas que se dirige al contra-electrodo con un potencial más bajo; en él las gotitas pierden el disolvente por evaporación quedando como especies iónicas en fase gaseosa. El contra-electrodo contiene un orificio a través del cual los iones alcanzan la cámara de vacío del espectrómetro en un proceso facilitado por la alta concentración de carga del mismo signo, positiva o negativa, atravesando regiones bombeadas diferencialmente mediante lentes selectoras ("skimmers") [76]. Los iones generados se transportan al analizador de masas mediante un campo eléctrico o magnético.

La ionización por EI se produce por interacción de las moléculas con los electrones energéticos emitidos por un filamento caliente de tungsteno o renio acelerados por un potencial de aproximadamente 70 V que se aplica entre el filamento y el ánodo. Las trayectorias de los electrones y las moléculas forman ángulo recto y se cruzan en el centro de la fuente, donde colisionan y tiene lugar la ionización. El producto primario son iones de una única carga positiva que se forman cuando los electrones de elevada energía se acercan suficientemente a las moléculas para causarles la pérdida de electrones por repulsión electrostática; por tanto, el producto o ión molecular es un ion radical que tiene el mismo peso molecular que la molécula [77]. Una vez que en la interfase se ha producido la transferencia de los iones, estos son dirigidos hacia el analizador de masas. Los analizadores de masas permiten la separación, detección y cuantificación de los analitos en estudio con una gran sensibilidad y selectividad, proporcionando información sobre su masa molecular.

En cuanto al espectrómetro de masas, existen diversos tipos disponibles comercialmente, pero en esta introducción sólo se describirán brevemente los más

utilizados en el análisis de muestras vegetales.

Un detector de masas de cuadrupolo simple consiste en cuatro barras paralelas de sección hiperbólica en su cara interna, generalmente de unos 15–20 cm de largo y 0.5 cm de radio, separadas entre sí unos 2 cm, a las que se aplica un potencial combinado de corriente continua y de radiofrecuencia que crea en su interior un campo denominado cuadrupolar (Fig. 3). Los iones generados en la fuente deben atravesar longitudinalmente el recinto limitado por estas barras para incidir en el detector. Estos iones, que entran en el analizador con una energía de unos pocos electronvoltios, se someten al efecto del campo cuadrupolar que los hace oscilar y los desvía en función de su relación *m/z* de forma que para una combinación de potenciales sólo los iones en un estrecho rango de valores *m/z* llegan a incidir en el detector. Los analizadores de cuadrupolo actúan por tanto como filtros de iones y los espectros de masas en estos sistemas se obtienen mediante un barrido de potencial aplicado a las barras. En consecuencia, en cada instante sólo se monitoriza una pequeña fracción del total de iones, mientras que el resto se desecha. Como los analizadores de cuadrupolo simple trabajan sólo con campos eléctricos, los barridos pueden ser extraordinariamente rápidos (0.01 s); por otra parte, no precisan rendijas para el enfoque del haz con lo que la sensibilidad del equipo aumenta significativamente. Otra ventaja es que la escala de masas es lineal con respecto a los potenciales utilizados, de forma que en los espectros obtenidos por este procedimiento pueden realizarse interpolaciones de masas con facilidad. El principal inconveniente que presenta este analizador es su poder de resolución, relativamente bajo —del orden de 500 a 1000— y su limitado rango de utilización, ya que sólo permite la separación de iones con relaciones *m/z* menores de 1000. Este tipo de espectrómetro de masas acoplado a un GC fue la herramienta analítica seleccionada para los estudios recogidos en los Capítulos VII y VIII de esta Memoria.

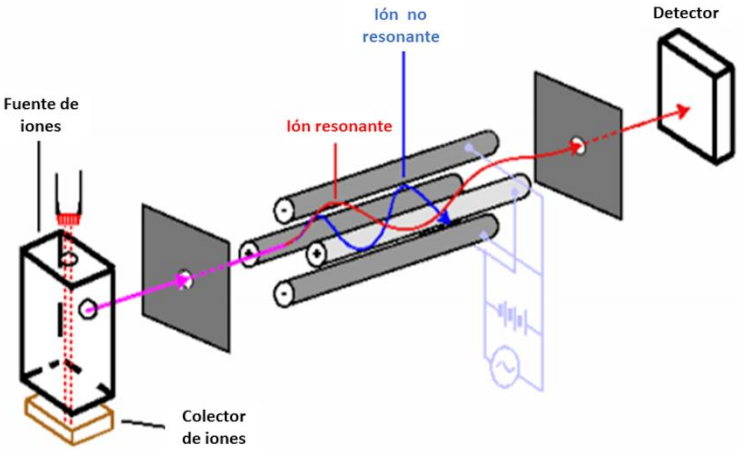

Figura 3. Esquema de un analizador de masas de cuadrupolo simple.

El analizador de trampa de iones (IT) permite el confinamiento de iones en una cámara de pequeño tamaño utilizando campos eléctricos o electromagnéticos. En este tipo de analizadores se pueden almacenar, seleccionar y analizar los iones formados en la misma trampa o en fuentes de ionización externas. Los iones pueden mantenerse en el interior de la trampa durante tiempos largos con objeto de favorecer la observación de descomposiciones metaestables o de fragmentos producidos por colisión de moléculas de gas. Los fragmentos iónicos generados en estos procesos pueden ser a su vez seleccionados de nuevo en la misma trampa de forma que el conjunto equivale a un sistema de espectrometría de masas en tándem múltiple. La Fig. 4 muestra un esquema de este tipo de analizador, en la que puede verse que está formado por tres electrodos de superficie hiperbólica, de los que el central es anular, mientras que los electrodos superior e inferior cierran los extremos del anillo. Los tres electrodos forman una cavidad en la que se produce la ionización, la fragmentación y el análisis de masas.

Durante el proceso de análisis de masas se aplica entre los electrodos superior e inferior un potencial de radiofrecuencia de 525 kHz (voltaje de modulación axial); al mismo tiempo, sobre el electrodo central se aplica otro voltaje de

radiofrecuencia de 1.1 MHz y de amplitud variable entre 0 y 7500 V. Estos dos voltajes de radiofrecuencia dan lugar a un campo electromagnético cuadrupolar tridimensional en el que quedan confinados los iones con una trayectoria oscilante estable, dependiendo el movimiento exacto de cada ion de los voltajes aplicados y de su relación *m/z*. Para detectar los iones se altera la señal de radiofrecuencia del electrodo anular, lo que da lugar a la desestabilización de la trayectoria de un ion concreto que es proyectado de la trampa. Un cambio gradual en la amplitud del campo de radiofrecuencia dará lugar a que los iones sean proyectados de la trampa en orden creciente de su relación *m/z*, lo que producirá un espectro de masas.

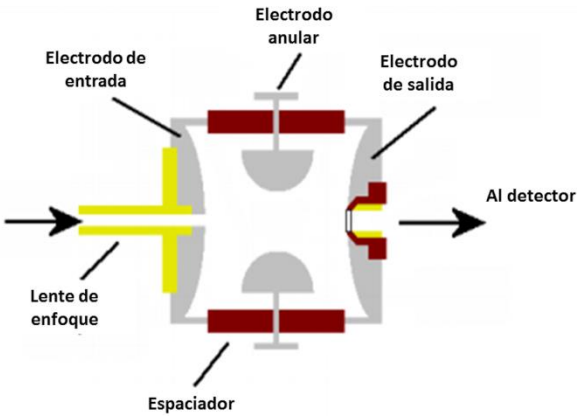

Figura 4. Esquema de un analizador de trampa de iones.

El analizador de IT presenta la ventaja de una sensibilidad mayor que las conseguidas con otros analizadores. Al mismo tiempo, su velocidad de barrido es también muy alta, por lo que sus prestaciones como detector cromatográfico son francamente buenas. Por otro lado, debe tenerse en cuenta que, al existir una concentración de iones bastante elevada en el interior de la trampa, es relativamente frecuente la existencia de reacciones bimoleculares entre los iones, lo que puede dar lugar a espectros con esquemas de fragmentación diferentes a los que se obtienen con otros tipos de analizadores.

Un detector de masas de triple cuadrupolo (QqQ) consta de las unidades que se muestran en la Fig. 5 [76]: una fuente de ionización (generalmente de tipo ESI) seguida de un conjunto de lentes para la transferencia de iones al primer cuadrupolo, formado por cuatro barras paralelas a las que se aplican voltajes específicos de corriente continua y de radiofrecuencia que hacen que todos los iones excepto los de uno o varios valores de *m/z* se filtren a su través eliminándose (Fig. 5). El voltaje aplicado es variable, por lo que secuencialmente unos iones pasan entre ellos y otros siguen circulando hasta alcanzar la celda de colisión en la que se fragmentan. Esta celda, que generalmente recibe el nombre de segundo cuadrupolo, es en realidad un hexapolo relleno de un gas inerte, nitrógeno o argón, en el que se fragmentan los iones, que se envían al tercer cuadrupolo para una segunda etapa de filtrado que permite aislar y examinar las múltiples transiciones desde el precursor al ión producto. Éste es el modo llamado monitorización de reacciones seleccionadas (selected reaction monitoring o SRM). Puesto que los fragmentos iónicos formados son partes de la molécula precursora, representan porciones de su estructura global. Estos detectores se utilizan preferentemente para análisis orientado, ya que permiten cuantificar con excelente sensibilidad y selectividad en el modo SRM. Estos fueron los motivos por los que se seleccionó este detector para los estudios cuantitativos recogidos en esta Memoria (Capítulos III, V y VI).

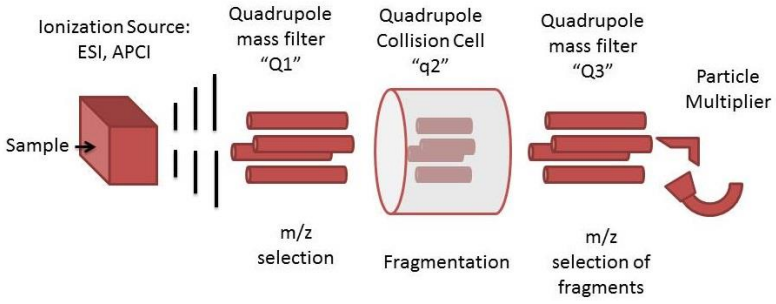

Figura 5. Esquema de un analizador de triple cuadrupolo.

El detector de masas del tipo cuadrupolo con tiempo de vuelo (QTOF) ha sido uno de los espectrómetros utilizado en la investigación recogida en los Capítulos II, IV, IX y X de esta Memoria. En la Fig. 6 se puede ver que este detector consta de una fuente de iones y un conjunto de lentes para la transferencia de iones al primer cuadrupolo, formado por cuatro barras paralelas a las que se aplican voltajes específicos de corriente continua y de radiofrecuencia para el filtrado de iones de *m/z* concreta con el fin de fragmentarlos en la célula de colisión [78]. El voltaje aplicado puede ser fijo para aislar un valor de *m/z* característico o variable para aislar de forma secuencial un rango de iones determinado con diferente valor de *m/z*. Los iones aislados se dirigen a la celda de colisión en la que se fragmentan mediante la aplicación de una energía de colisión. Esta celda, que recibe el nombre de segundo cuadrupolo, generalmente se trata de un hexapolo en el que se inyecta un gas inerte, nitrógeno o argón, como gas de colisión para fragmentar los iones, que se envían al tubo de tiempo de vuelo. A los iones que llegan al TOF se les aplica un pulso de energía constante de forma que recorren el tubo a una velocidad inversamente proporcional a su relación *m/z,* lo que produce la separación entre ellos, su llegada al detector y la generación del espectro correspondiente. La longitud del tubo de vuelo establece la resolución espectral ya que, a mayor longitud, mayor resolución espectral podrá conseguirse.

Comparado con el analizador de QqQ, el QTOF ofrece una mayor selectividad que el primero, aunque la sensibilidad es considerablemente menor. Por tanto, el QTOF está orientado al análisis cualitativo o semicuantitativo, mientras que el QqQ encuentra su principal área de aplicación en el análisis cuantitativo y confirmatorio. El QTOF puede funcionar en modo MS con el TOF como herramienta de análisis aprovechando la alta precisión en masa, o en modo MS/MS para la elucidación estructural. Por otra parte, y por su exactitud en la medida de masas (error menor de 2 ppm), se puede llevar a cabo una muy buena identificación, lo que lo convierte en una herramienta adecuada para análisis

global.

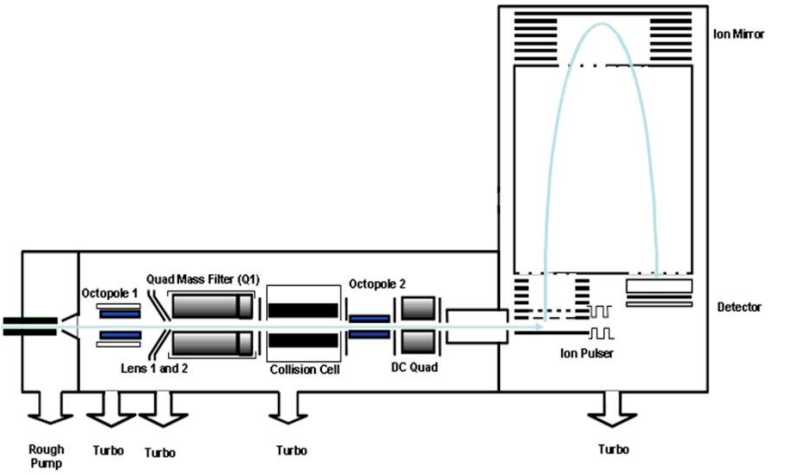

Figura 6. Esquema general de un espectrómetro de masas de tiempo de vuelo de cuadrupolo.

5. Análisis de datos

El análisis de datos es de gran importancia en química analítica ya que permite transformar los datos brutos en información útil para el estudio. Para conseguir ese tipo de información se emplean herramientas quimiométricas y bioinformáticas en el tratamiento de los datos brutos [79]. El análisis de los datos generados en estudios de muestras vegetales, especialmente en los que se pretende obtener información cualitativa, es complejo y requiere varias etapas, tal como se esquematiza en la Fig. 7.

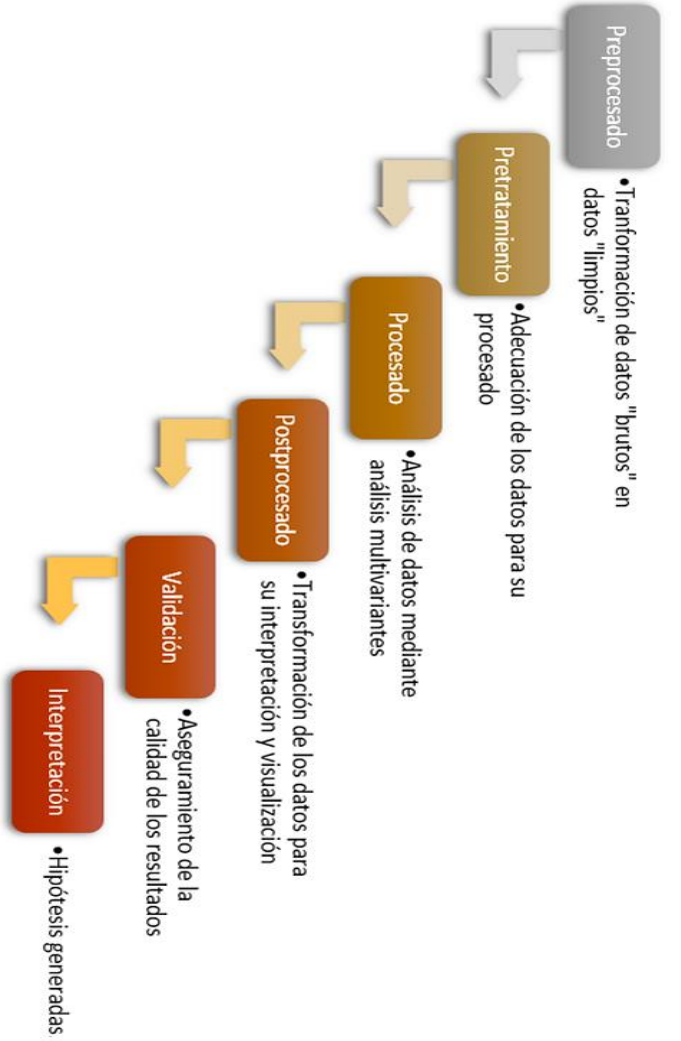

Figura 7. Esquema de las etapas del análisis de los datos.

5.1. Preprocesado de datos

La finalidad del preprocesado de datos es la transformación de los datos "brutos" (los que proporciona el instrumento analítico y que son exclusivos de la marca comercial) en datos "limpios" (aquéllos que poseen un formato universal) para su procesado por cualquier software de tratamiento de datos. Las principales técnicas de preprocesado de datos son:

- Deconvolución, que permite resolver *in silico* señales solapadas en NMR o GC–MS y LC–MS. A partir de los datos cromatográficos se genera una tabla de señales donde cada metabolito se representa por una variable.
- Alineamiento, mediante el que se ordenan las señales correspondientes a los mismos metabolitos en registros NMR o en cromatogramas para que tengan el mismo desplazamiento químico o el mismo tiempo de retención, ya que son valores que pueden variar entre análisis. Es una herramienta muy útil cuando el estudio requiere la combinación de datos procedentes de diferentes muestras.
- Corrección de la línea de base, para la eliminación del ruido de fondo en registros NMR y en cromatogramas, así como de señales no asociadas a metabolitos [80]. El ruido de fondo puede ser químico, debido al proceso de medida, o aleatorio, asociado al sistema de detección.
- Corrección de fase, que se utiliza para eliminar la asimetría de los picos en los espectros NMR obtenidos por transformada de Fourier.
- Selección de picos (peak-picking), que organiza las señales obtenidas mediante NMR o MS y las presenta como tabla para su posterior tratamiento [81].

5.2. Pretratamiento de datos

Los datos obtenidos anteriormente se presentan normalmente como una tabla que contiene los resultados analíticos a los que se aplican diferentes

operaciones matemáticas para facilitar la comparación entre muestras. Las operaciones aplicadas con mayor frecuencia con este propósito son:

- Normalización, que permite estandarizar los datos para hacerlos comparables mediante el ajuste del ruido.
- Escalado, que establece la intensidad de las diferentes señales en función de la señal más intensa con el fin de facilitar la comparación entre ellas.
- Centrado, que traslada el centro de gravedad de un archivo de datos y elimina la importancia de determinadas señales.
- Detección de valores anómalos (outliers), que identifica las variables, muestras o combinaciones de ambas que se desvían de la distribución normal de la mayoría de los datos.

5.3. Procesado de datos

Los estudios de muestras agroalimentarias pueden generar una gran cantidad de datos que dependen de un número grande de variables. El procesado permite aplicar a estos datos análisis univariantes y multivariantes para conseguir información útil de ellos [82]. Al estar implicadas muchas variables, el análisis multivariante es el más usado en la gran mayoría de los casos, e integra un conjunto de técnicas estadísticas cuya finalidad es analizar de forma simultánea un conjunto de datos formados por varias variables medidas en cada muestra. Sus objetivos son: (i) visualizar los datos, es decir, conseguir detectar relaciones entre las diferentes muestras usando gráficas, animaciones o imágenes; (ii) analizar datos para buscar pautas consistentes o relaciones entre las variables que componen un conjunto de datos complejo; y, (iii) modelar los datos para determinar relaciones entre conceptos y objetos mediante la preparación de un modelo matemático. Las técnicas de análisis multivariante se clasifican en dos grupos según su finalidad:

- Análisis no supervisado (exploratorio), para poner de manifiesto la

existencia de conjuntos (clusters) de muestras y detectar cambios locales de densidad de datos e identificar valores anómalos y/o fuentes de variabilidad de los datos. Los algoritmos utilizados en este caso no requieren *a priori* ningún tipo de información adicional de las muestras y están basados en el cálculo de la semejanza o no entre las muestras. Los análisis no supervisados más frecuentemente utilizados son el análisis por componentes principales (PCA) y el análisis de conjuntos o clústers (CA) [83].

- Análisis supervisado (clasificatorio), con el que se desarrollan modelos que permiten predecir la clasificación de muestras desconocidas. Puede ser de tipo cualitativo (si se predice la clase a la que pertenece una serie de muestras) y/o cuantitativo (si se establecen probabilidades de clasificación y se definen los límites de cada clase y factores de respuesta cuantitativos para un amplio rango de muestras). En este caso es necesario obtener información para el desarrollo del modelo. También se requiere una etapa de validación del modelo ya que existe probabilidad de correlaciones entre los descriptores del modelo y una amplia dimensionalidad. Los análisis supervisados más utilizados son los basados en mínimos cuadrados parciales (PLS) y en "soft independent modeling class analogy" (SIMCA) [84].

5.4. Postprocesado de datos

El objetivo del postprocesado de los datos es interpretar los análisis llevados a cabo en la etapa anterior, con lo que se pretende conocer los metabolitos causantes de la diferenciación entre muestras y estudiar sus cambios químicos y de concentración en las muestras. Estos metabolitos se denominan marcadores y la monitorización de su concentración permite estudiar la evolución de una planta durante su crecimiento o de un alimento durante su conservación, detectar factores externos que les puedan afectar (como factores climatológicos,

fertilizantes, sistemas de conservación y envasado, etc.) o monitorizar la evolución durante el procesado de un alimento.

5.5. Validación de los resultados

En la validación de los resultados se corrobora que los marcadores previamente identificados lo han sido correctamente. Para ello, existen bases de datos de metabolitos en las que se aportan sus datos químicos y biológicos, así como sus espectros de fragmentación en MS. La comparación de los datos obtenidos en el análisis de las muestras con los datos existentes en estas bases de datos permite confirmar que la identificación ha sido correcta. Algunas de las bases de datos más empleadas son la METLIN, la Human Metabolome Database (HMDB), la Food Database (FooDB), la MassBank o la MS Database. Otra alternativa para la validación de los resultados es la comparación de los metabolitos identificados con sus patrones comerciales, que es una validación más fiable, ya que no influyen variables instrumentales. Sin embargo, presenta el incoveniente de la dificultad de encontrar patrones comerciales de compuestos poco comunes en matrices vegetales. La validación es especialmente importante en los estudios que pretenden caracterizar el perfil composicional de una muestra vegetal, ya que permite identificar de forma individual los compuestos del perfil.

6. La metabolómica como marco de estudio de muestras vegetales

El auge de la metabolómica en la última década ha hecho que esta disciplina adquiera un papel cada vez más importante en estudios sobre la elucidación de mecanismos, rutas y comportamiento de seres vivos, así como en estudios sobre su interacción con el medio. En sus comienzos, esta nueva "ómica" fue definida por Fiehn (2002) como "un análisis global, exhaustivo en el que se identifican y cuantifican todos los metabolitos de un sistema biológico". Desde entonces, la metabolómica se ha definido de diferentes formas en numerosos artículos y

revisiones científicas, por lo que Beyoğlu and Idle (2013) aunaron todas ellas en una definición amplia de la nueva disciplina como "el estudio global y no sesgado del conjunto de moléculas pequeñas (<1 kDa) en un biofluido, tejido, órgano u organismo" [86]. El conjunto de moléculas pequeñas conforma el metaboloma, definido como "la serie completa de todos los metabolitos formados por una célula en respuesta a su metabolismo, comprendiendo el endometaboloma (todos los metabolitos intracelulares) y el exometaboloma (todos los metabolitos excretados al fluido extracelular)". El metabolismo, en definitiva, es una red extensa de reacciones metabólicas donde los productos de una reacción son los reactantes de otra subsiguiente. Por ello, se define metabolito como "cualquier intermedio o producto final del metabolismo, usualmente restringido a pequeñas moléculas que no están genéticamente codificadas". En función tanto de las rutas metabólicas en las que están implicados los metabolitos como de su función se pueden clasificar en:

- Metabolitos primarios: son aquéllos que están directamente implicados en el crecimiento, desarrollo y reproducción de un organismo.
- Metabolitos secundarios: son los que no están directamente implicados en los procesos de crecimiento, desarrollo y reproducción, pero generalmente tienen una función ecológica importante como, por ejemplo, la defensa contra predadores, parásitos o enfermedades, la competencia entre especies o la implicación en los procesos de reproducción (colores, olores, etc.).

El metaboloma, y por tanto el estado metabólico de un ser vivo, puede verse afectado por diversos factores intrínsecos (edad, estado de salud, estado reproductivo, etc.) y extrínsecos (nutrientes, compuestos artificiales como pesticidas, fertilizantes, contaminantes medioambientales, etc.). Por ello, el estudio del metaboloma es de gran importancia para conocer el estado de un organismo y cómo le afecta su entorno.

La metabolómica está directamente relacionada con el resto de "ómicas" como consecuencia del flujo de información biológica (Fig. 8), descrito por Gomase *et al.* [87]. Así, la genómica es la ómica que estudia el ADN de un organismo o célula, es decir, su carga genética o su genotipo. El siguiente eslabón en el flujo de información es el ARN mensajero, que estudia la transcriptómica y que soporta la información para el estudio de las proteínas, objeto de la proteómica. El objetivo de la última de las grandes "ómicas" es estudiar los metabolitos que resultan de la acción enzimática de ciertas proteínas. El metaboloma está considerado la última expresión de la información biológica, por lo que se relaciona directamente con el fenotipo. Como ejemplos se pueden considerar los pigmentos, que afectan al color de las flores, hojas y frutas, o la acumulación de azúcares, que proporciona el sabor dulce a la fruta. La información obtenida a partir de todas o algunas de estas "ómicas" puede combinarse para conseguir un conocimiento más completo de la biología de un organismo y, por tanto, de los procesos que afectan al crecimiento, envejecimiento, reacciones a estímulos externos, etc. El uso de la información combinada proveniente de varias "ómicas" constituye la biología de sistemas o integración de ómicas.

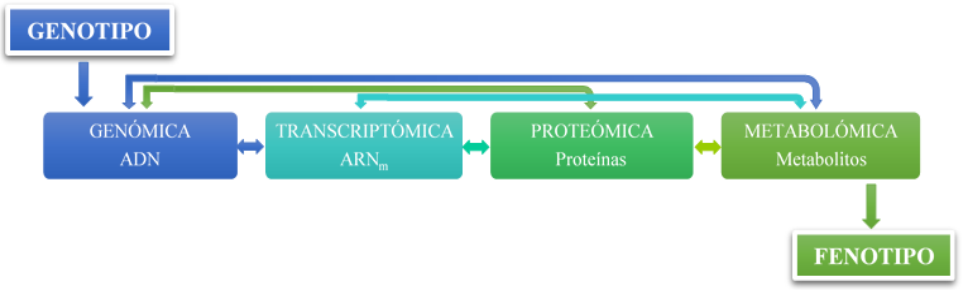

Figura 8. Relación entre las grandes "ómicas".

La metabolómica presenta importantes ventajas para el estudio de un sistema biológico con respecto al resto de "ómicas". Para llevar a cabo este tipo de estudios se utilizan plataformas analíticas que se caracterizan por tener una excelente

reproducibilidad analítica y biológica con un bajo coste del análisis por muestra, en contraposición a los costes de la aplicación de cualquier otra de las grandes "ómicas". Sin embargo, esta disciplina presenta ciertas características que la hacen más compleja que otras "ómicas" en algunos aspectos. Los metabolitos son moléculas muy diversas químicamente, no se encuentran confinados, como en el caso del ADN, y están presentes en el organismo en un gran número (un sistema vegetal puede alcanzar hasta los 200.000 metabolitos) y en un amplio rango de concentraciones que puede abarcar hasta 9 órdenes de magnitud (de picomoles a milimoles). La gran variedad de los metabolitos y su amplio rango de concentraciones hacen necesaria la aplicación de técnicas muy diversas, tanto para la preparación de la muestra, mencionadas en el apartado 2 de esta introducción, como para el análisis de los metabolitos en cuestión, descritas en el apartado 3. La experiencia del investigador juega un papel clave en la selección de las técnicas más adecuadas en función de la naturaleza de los metabolitos a determinar y de la matriz de la muestra.

6.1. Estrategias analíticas en metabolómica y sus principales objetivos

Existen distintas estrategias analíticas en metabolómica entre las que se puede elegir dependiendo del objetivo del estudio [88]. En general todas ellas se caracterizan por un coste menor de análisis por muestra y tiempos de análisis más cortos que las otras grandes "ómicas". Las principales de estas estrategias son las siguientes:

Huella metabolómica o "metabolomic profiling"

El perfil metabolómico se utiliza para clasificar muestras basándose en una característica determinada (procedencia, relevancia u origen biológico). Se utilizan espectros de NMR o de MS del conjunto de metabolitos en estudio para obtener un conjunto de señales características. Es una estrategia rápida, pero los datos obtenidos son genéricos, ya que no aporta información específica de los metabolitos, por lo que se orienta a la clasificación rápida de numerosas muestras

utilizando estadística multivariante. Un estudio de este tipo fue llevado a cabo por Pan *et al.*, que analizaron la maca (*Lepidium meyenii*) cultivada en distintas zonas geográficas mediante la obtención de la "huella" generada por los compuestos denominados macamida [89]. Un PLS que recoge todas las muestras analizadas mostró una separación clara entre zonas geográficas debida a las diferencias en la "huella" obtenida para cada tipo de muestra (Fig. 9).

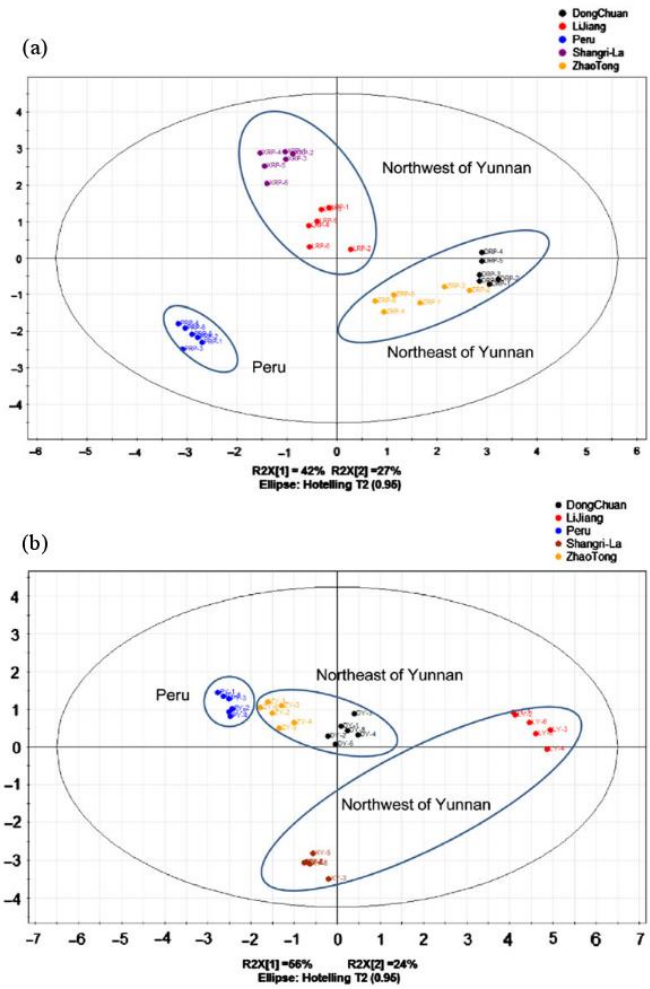

Figura 9. Gráfico de residuales del PLS de maca roja y morada (a) y maca amarilla (b) procedente de diferentes zonas geográficas. Con permiso de John Wiley and Sons.

Análisis orientado (dirigido) o "targeted analysis"

El análisis orientado se restringe a un número limitado de metabolitos (a veces un único metabolito) que han sido caracterizados previamente; por lo que representa una pequeña fracción del metaboloma. La cuantificación de metabolitos clave con funciones bien definidas en rutas metabólicas puede usarse para la detección de perturbaciones de estas rutas, lo que puede dar información sobre el estado general del sistema en estudio. El análisis orientado, al estar enfocado a un grupo reducido de metabolitos, generalmente requiere una etapa de preparación de la muestra generalmente laboriosa como, por ejemplo, una SPE. Ciertos autores argumentan que el análisis orientado no puede considerarse una estrategia de la metabolómica, sino un mero análisis de un número limitado de compuestos. No obstante, la selección de esos compuestos para realizar el seguimiento de un proceso metabólico (o para la discriminación entre diferentes comportamientos metabólicos) constituye estrictamente un estudio metabolómico. Esta fue la estrategia usada en los estudios recogidos en los Capítulos III, V y VI para el análisis de un número reducido de compuestos (flavonoides procedentes de la piel de naranja en el Capítulo III y glicósidos de esteviol y compuestos fenólicos en la hoja de estevia rebaudiana en los Capítulos V y VI, respectivamente).

Análisis metabolómico global o "untargeted analysis"

Recibe el nombre de análisis metabolómico global el análisis cualitativo y/o cuantitativo de todos los metabolitos o gran parte de ellos presentes en un sistema. Es una estrategia que en ciertas ocasiones requiere la integración de los resultados obtenidos por aplicación de diferentes métodos de preparación de la muestra y diferentes métodos de análisis basados en diferentes técnicas de separación y de detección. Además, el conjunto de datos obtenidos mediante aplicación de estos métodos es muy extenso, por lo que requiere tratamientos con técnicas quimiométricas avanzadas para su conversión en datos manejables y, finalmente,

en resultados interpretables. Los metabolitos se identifican mediante dos vías de comparación: con patrones o con los datos procedentes de bases de datos especializadas (preferiblemente mediante ambas para una identificación segura). Esta estrategia permite la identificación de nuevos metabolitos que estén implicados en rutas metabólicas y, por tanto, el conocimiento de esas rutas. Para el desarrollo de la investigación recogida en gran parte de los capítulos de esta Memoria se seleccionó esta estrategia con el fin de obtener la mayor información posible de los compuestos que forman parte de la piel de naranja (Capítulo II), de la hoja de estevia rebaudiana (Capítulo IV) y de la fracción volátil (Capítulos VII y VIII) y polar (Capítulos IX y X) del ajo fresco y del ajo negro.

6.2. Pretratamiento y preparación de muestras vegetales en metabolómica

La selección de las técnicas de pretratamiento y preparación de las muestras vegetales en estudios metabolómicos depende estrechamente del objetivo del estudio y de la estrategia analítica a seguir.

En estudios en los que se pretende obtener la huella metabolómica o realizar un análisis metabólico global, el pretratamiento y la preparación de la muestra deben ser simples con el fin de evitar pérdidas de cualquier fracción de compuestos presentes en ella, como puede ser la fracción volátil. En contraposición, cuando se trata de un análisis orientado los protocolos de pretratamiento y preparación de la muestra deben ser selectivos ya que es necesario obtener la fracción deseada y eliminar o minimizar la presencia de compuestos que puedan interferir en la medida de los compuestos de interés. Por ello, en estos casos generalmente se requiere un mayor número de etapas en la preparación de la muestra.

Además, hay que tener en cuenta que en los estudios metabolómicos es muy importante incluir etapas de pretratamiento de la muestra que interrumpan el metabolismo. Esta etapa debe ser muy rápida, compatible con las demás etapas

del proceso analítico y no inducir cambios químicos en los metabolitos. Por ello, las técnicas más empleadas con este objetivo son la disminución de la temperatura hasta un valor entre −20 y −80 ºC o el uso de valores extremos de pH. También es esencial la eliminación de agua de la muestra ya que es el medio donde se producen las reacciones metabólicas; siempre teniendo en cuenta que ciertos procedimientos de eliminación de agua pueden inducir cambios composicionales en la muestra y, por tanto, modificar el metaboloma de la muestra. Esto se ha demuestrado en el Capítulo III de esta Memoria, en el que se comparan las técnicas de secado en estufa y liofilización para la eliminación de agua de la piel de naranja, obteniendo diferencias en la concentración de flavonoides en función de la técnica usada.

Las técnicas de extracción generalmente empleadas en metabolómica son las comentadas en el apartado 3 de esta introducción, de las que se deberá seleccionar la idónea para el estudio metabolómico en cuestión. Para el análisis metabolómico global o de la huella metabolómica se seleccionará un protocolo de extracción que abarque el mayor número posible de los metabolitos presentes en la muestra. Por tanto, se deberá seleccionar una técnica de extracción simple y utilizar una mezcla de extractantes de distinta polaridad. Como ejemplo, se considera el estudio comparativo del perfil metabolómico de la planta *Nicotiana langsdorffii* salvaje y transgénica para evaluar su respuesta al estrés. La etapa de pretratamiento de la muestra implicó liofilización, molienda y homogeneización; mientras la preparación de la muestra consistió en la extracción de los metabolitos en un baño de ultrasonidos usando como extractante una mezcla metanol–agua 75:25 (v/v). Este protocolo permitió detectar 200 metabolitos e identificar los metabolitos secundarios afectados por las condiciones de estrés inducido a la planta [90].

El análisis metabolómico orientado requiere seleccionar un protocolo de extracción que sea lo más selectivo posible para los compuestos a determinar, que suele caracterizarse por incluir varias etapas que permiten la extracción selectiva

de los compuestos y la eliminación de interferentes. Es el caso de los protocolos empleados por Rojano-Delgado *et al.* para la extracción de glufosinato y sus metabolitos en trigo harinero (*Triticum aestium*). Se emplearon 3 técnicas de extracción (convencional, asistida por microondas y asistida por ultrasonidos), que necesitaron en todos los casos etapas adicionales de limpieza para la eliminación de polisacáridos, proteínas y péptidos que actúan como interferentes; así como la preconcentración de los metabolitos a determinar [91].

6.3. Herramientas analíticas empleadas en metabolómica

Las herramientas analíticas empleadas en estudios metabolómicos de muestras vegetales son la NMR y, especialmente, la MS acopladas a técnicas de separación previas. Son éstas las herramientas analíticas que actualmente proporcionan una mayor información sobre los metabolitos que constituyen la muestra, especialmente la MS debido a las ventajas de sensibilidad y selectividad que presenta frente a la NMR. En estudios metabolómicos pueden emplearse todos los detectores de MS explicados en el apartado 4 de esta introducción, sin embargo, la clave está en la elección del adecuado en función de la estrategia a seguir y de las características de los metabolitos.

En estudios de la huella metabolómica puede emplearse prácticamente cualquier detector ya que solo se tiene en cuenta el conjunto de señales obtenidas; sin embargo, en estos estudios se usa frecuentemente la NMR debido a la gran cantidad de señales que aporta y a la ventaja de no necesitar una preparación de muestra compleja. Por otro lado, el detector QTOF es el más empleado para los estudios metabolómicos globales, lo que se debe a que presenta una gran exactitud en la medida del valor de *m/z*, permitiendo así una identificación fiable de los metabolitos mediante comparación con los espectros recogidos en las bases de datos. Por último, los estudios orientados requieren detectores que permitan detectar los metabolitos en bajas concentraciones y evitando interferentes que puedan enmascarar la medida de los analitos de interés. Por ello, el más empleado

en estos estudios es el QqQ debido a que la selección de la transición mediante el método SRM focaliza la detección exclusivamente en los compuestos de interés y proporciona una excelente sensibilidad, pudiendo detectar metabolitos en concentraciones del orden de los ng/mL.

En resumen, cuando se emplea la metobolómica para el estudio de muestras vegetales todo el proceso analítico queda supeditado a la estrategia metabolómica seleccionada, según se esquematiza en la Fig. 10.

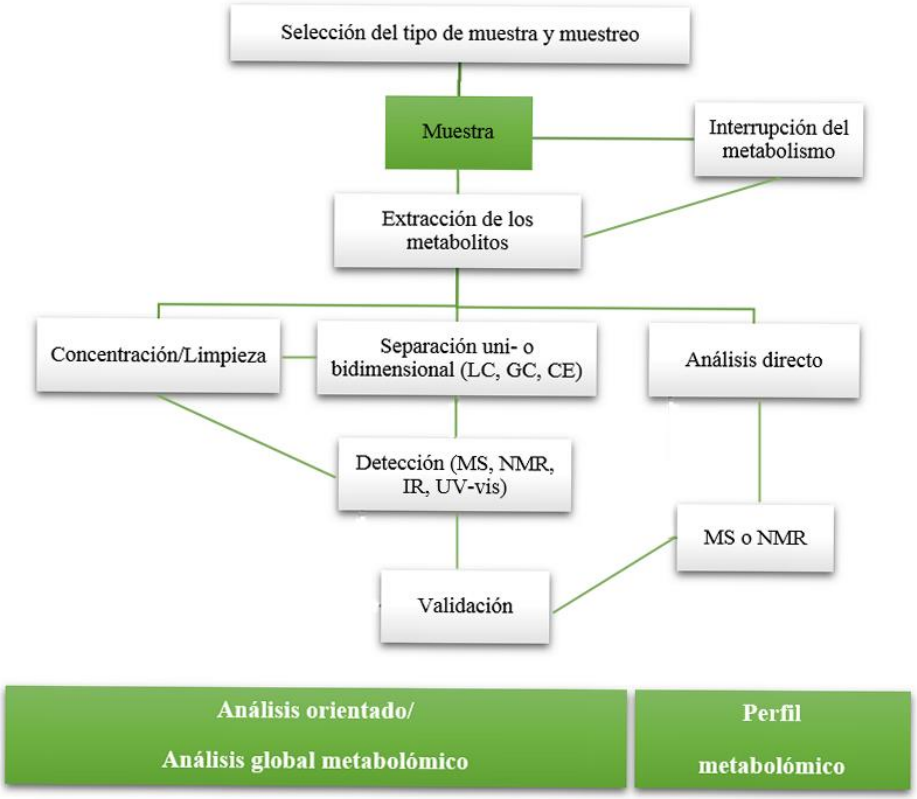

Figura 10. Diagrama de flujo del proceso analítico en función de la estrategia metabolómica.

7. Algunos de los retos pendientes en metabolómica vegetal

La metabolómica vegetal tiene planteados retos que se deberían afrontar y que derivan de una investigación mal planteada y con escasos recursos instrumentales en la mayoría de los casos.

Una de estas líneas de investigación, que se caracteriza por un número desorbitado de publicaciones, se dedica a la búsqueda de compuestos (antioxidantes mayoritariamente) en plantas y en los residuos de la industria agroalimentaria. A pesar de que se pretende englobar estos estudios en la metabolómica, realmente no implican aspectos metabólicos. Ejemplo de este comportamiento es la proliferación masiva de métodos para la extracción de metabolitos utilizando energías auxiliares y en los que sólo se determina la eficiencia. Para ello se realiza una medida global en los extractos de los compuestos que responden al detector (e.g., medida a una determinada longitud de onda de la absorbancia intrínseca de los compuestos o de sus productos de derivatización). Una investigación racional en esta línea debería implicar:

- La distinción clara de los compuestos de interés (e.g., compuestos antiinflamatorios, anticancerígenos) y el uso de la instrumentación adecuada en el seguimiento del proceso de extracción para optimizar su eficiencia respecto a esos compuestos y las condiciones de la planta en cuestión para favorecer el metabolismo que dé lugar a una mayor concentración de estos compuestos.
- Una comprobación exhaustiva de la ausencia de degradación de los metabolitos por la acción de energías auxiliares, o incluso por efecto prolongado de la maceración, la agitación o la temperatura. Comprobada la degradación, la optimización del proceso debe contemplar la ausencia de este fenómeno indeseable.
- La investigación de otros componentes diferentes de aquéllos considerados como únicos de interés en una determinada planta. Como

ejemplo, en la estevia rebaudiana se han investigado únicamente sus propiedades edulcorantes, por lo que los estudios sobre esta planta han estado exclusivamente orientados a los glicósidos de esteviol, a pesar de que sus propiedades saludables no pueden atribuirse a estos compuestos. Un paso adelante en este caso se ha dado en la investigación recogida en el Capítulo VI al extraer e identificar los compuestos fenólicos y determinar las variedades de estevia rebaudiana más ricas en estos compuestos.

- La interpretación de la función en la planta de los compuestos extraídos. En general se trata de metabolitos secundarios que se forman en la planta como mecanismo de defensa; acción que pueden ejercer individualmente o ser el resultado del efecto simbiótico de varios metabolitos. Conocer su función en la planta es la premisa para aprovechar el efecto que pueden causar en animales y en humanos y favorecer su formación.
- El estudio en animales y después en humanos del efecto y de las dosis adecuadas de los compuestos extraídos, ya que en la mayoría de los casos el fin último es el uso de estos compuestos como suplementos nutricionales o como nutracéuticos. Este estudio entroncaría con la medicina personalizada, un área cuyo desarrollo está todavía en sus inicios, pero que debería abordar este tipo de estudios como una de las vías de sostenibilidad en relación con la valorización de residuos.

Los nuevos productos que se comercializan por la industria agroalimentaria hacen imprescindible un estudio exhaustivo de los compuestos que se mantienen de aquéllos que existían en la materia prima, los nuevos compuestos que se han formado y el efecto de ambos en el organismo al que van destinados. En esta Tesis Doctoral (Capítulos VII, VIII, IX y X) se recoge uno de estos estudios, en este caso del ajo negro.

Los estudios de cruzamientos de distintas especies o variedades de plantas,

así como las genéticamente modificadas constituyen un campo complejo y de gran interés en el que a la metabolómica no se le ha dado la importancia que realmente merece. El efecto que los cambios genéticos producen en el metabolismo de la planta cruzada o modificada es fácilmente cuantificable. Tal es el caso del estudio recogido en el Capítulo VI de esta Memoria, donde se cuantifican compuestos fenólicos en plantas de estevia rebaudiana, algunas de ellas generadas mediante cruzamiento de variedades. La genética de estos híbridos o transgénicos se sigue estudiando más profusamente mediante genómica [92] o transcriptómica [93], aunque el mayor interés está en los cambios que se producen en los metabolitos, que constituyen la parte más valiosa de la planta con vistas a su resistencia a enfermedades o condiciones climatológicas adversas, así como a uso medicinal o nutricional. La amplificación del fenómeno transgénico "corriente abajo" proporciona información en muchas ocasiones no detectable mediante las técnicas analíticas generalmente utilizadas en genómica o transcriptómica y cuyos análisis resultan enormemente más caros.

Finalmente, el metabolismo de pesticidas y herbicidas es muy poco conocido, a pesar de que constituye una parcela de enorme interés. La asimilación de estos compuestos por parte de la planta puede dar lugar a metabolitos que pueden ejercer un efecto muy diferente al del producto de partida, tanto en las plantas como en el suelo que finalmente los recibe. En el caso de los herbicidas, los metabolitos pueden hacer las hierbas resistentes al herbicida o ser perjudiciales para la planta o para los metabolitos que interesan en ella. Los estudios realizados por el grupo en el que se integra la doctoranda sobre el metabolismo del glifosato [94], el glioxilato [95], el imazamox [96] y el glufosinato [91] constituyen una llamada de atención sobre el interés de la investigación en esta área.

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HERRAMIENTAS Y EQUIPOS ANALÍTICOS

En este apartado de la Memoria se describen someramente los diferentes tipos de muestras que han sido objeto de la investigación, los instrumentos y aparatos utilizados durante el desarrollo experimental de la Tesis, así como las herramientas quimiométricas y las bases de datos. En los diferentes capítulos se incluye una explicación más detallada de los que se han usado en la investigación recogida en cada uno de ellos.

1. Muestras

Para el desarrollo de la investigación que se recoge en la Parte A se ha usado un único tipo de muestra: piel de naranja. Para la que constituye el Capítulo III se utilizó piel procedente de ocho variedades de naranja.

Para la Parte B se utilizaron hojas secas de estevia rebaudiana procedentes de la empresa Vitrosur S.L., que en el Capítulo V provenían de plantas cultivadas en tres ambientes diferentes (en laboratorio, en invernadero y en campo), mientras que para el estudio recogido en el Capítulo VI se utilizaron hojas de cinco variedades diferentes de esta planta.

Por último, en la Parte C se utilizaron muestras de ajo fresco y ajo negro de 3 variedades diferentes, así como muestras de ajo en diferentes puntos del proceso de fermentación. En el Capítulo VII se utilizaron solamente las muestras de ajo fresco, mientras que en el Capítulo IX se usaron tanto muestras de ajo fresco como de ajo negro. Por último, el ajo obtenido en diferentes puntos de fermentación se usó para los estudios recogidos en los Capítulos VIII y X.

2. Sistemas para la preparación de muestra

Debido a que la mayoría de los estudios recogidos en esta Memoria se han orientado a la obtención de un perfil composicional lo más completo posible, se desarrollaron métodos con mínima o nula preparación de muestra. Con respecto a los estudios cuantitativos, se demostró que los compuestos fenólicos, flavonoides y glicósidos de esteviol eran fácilmente extraíbles, por lo que la preparación de la muestra no fue compleja. Los sistemas utilizados para la preparación fueron los siguientes:

Liofilizador. Se utilizó un liofilizador FTS en los Capítulos II y III para la eliminación de agua de la piel de naranja.

Molino ciclónico. Se usó un molino ciclónico de la marca Tecator para la trituración de la piel de naranja y la homogeneización del tamaño de partícula en el Capítulo II.

Triturador. Se empleó un triturador comercial para la homogeneización de la muestra en los Capítulos III, IV, V y VI.

Dispositivos de ultrasonidos. Existen dos tipos de dispositivos basados en ultrasonidos que se usan comúnmente en el laboratorio analítico: los baños y las sondas. Los primeros están presentes en cualquier laboratorio para la limpieza de material de vidrio y para la desgasificación de fases cromatográficas. Son dispositivos que presentan poca uniformidad en la aplicación de ultrasonidos y una caída progresiva de potencia con el tiempo, lo que los convierte en un sistema poco reproducible para su uso en un protocolo de extracción. Por ello, se seleccionó un dispositivo con sonda que permite tanto la aplicación directa sumergiendo la sonda en el sistema muestra−extractante, como indirecta usando un baño de agua para transmitir este tipo de energía. El sonicador utilizado fue un Branson 450 digital equipado con una sonda cilíndrica de aleación de titanio con 12.7 mm de diámetro. Se empleó para la extracción con emulsión asistida por

ultrasonidos llevada a cabo en el Capítulo II.

Agitador. Para favorecer la extracción sólido-líquido se utilizó un agitador Vortex digital IKA MS3 en la extracción mediante maceración con agitación en los Capítulos III, IV, V, VI, IX y X.

Espacio de cabeza. Para la extracción de compuestos volátiles (Capítulos VII y VIII) se empleó un automuestrador de espacio de cabeza Agilent 7694E. Este sistema está compuesto por un horno que permite calentar la muestra para la liberación de los compuestos volátiles, un muestreador del espacio de cabeza generado en el vial y una línea de transferencia termostatizada que permite el paso de los volátiles al sistema de separación al que se acopló este dispositivo.

Centrifugadora. Se empleó una centrifugadora de la marca Selecta para la ruptura de la emulsión y la separación de fases, así como del residuo sólido en el Capítulo II.

Concentrador. Se utilizó un concentrador 5301 de la marca Eppendorf para la preconcentración del extracto no polar en el Capítulo II.

3. Sistemas de separación y detección empleados

Los métodos desarrollados en la parte experimental de esta Tesis Doctoral se han basado en una separación cromatográfica (mediante LC o GC) y la posterior detección basada en absorción molecular o en espectrometría de masas.

En los estudios basados en la identificación del perfil polar y no polar de piel de naranja (Capítulo II) y de estevia rebaudiana (Capítulo IV), así como en la comparativa del perfil polar del ajo fresco y negro (Capítulo IX) y su evolución durante la fermentación (Capítulo X) se utilizó un equipo HPLC Agilent 1200 Series acoplado a un detector de masas de tiempo de vuelo de alta resolución Agilent 6540, realizando la detección en el modo MS/MS. También en el Capítulo

II se empleó un cromatógrafo de líquidos Agilent 1100 conectado a detector de diodos en fila para el estudio de optimización del proceso de extracción.

Para la cuantificación de flavonoides en piel de naranja (Capítulo III) y de glicósidos de esteviol y compuestos fenólicos en hojas de estevia rebaudiana (Capítulos V y VI) se utilizó un equipo Agilent 1200 Series acoplado a un espectrómetro de masas con triple cuadrupolo Agilent 6460 equipado con una fuente de ionización de electrospray con tecnología "Jet Stream".

En los casos anteriores se empleó una columna de fase reversa C18 para la separación de los compuestos. También en todos ellos se usó el software MassHunter para la adquisición de espectros y el análisis de los datos.

Por último, para la caracterización de perfil volátil de ajo fresco y negro (Capítulos VII y VIII) se empleó un cromatógrafo de gases Agilent 7890B acoplado a espectrómetro de masas de cuadrupolo simple Agilent 5977A y una columna capilar de sílice fundida Factor VF-5ms.

4. Herramientas quimiométricas

En la investigación recogida en esta Memoria se han empleado herramientas quimiométricas para el desarrollo y optimización de los métodos analíticos, así como para el tratamiento de los datos multivariantes obtenidos.

Se utilizaron distintos programas informáticos para el tratamiento de datos en función del objetivo perseguido:

- Alineamiento de entidades moleculares en análisis no orientado de las muestras (Capítulos II, IV, VII, VIII, IX y X), para el que se emplearon los softwares Qualitative Workstation y Mass Profiler Professional (Agilent). El primero se usó para la extracción de las entidades moleculares potenciales incluyendo los diferentes aductos e isótopos registrados, mientas que el

segundo permitió el alineamiento de las entidades en las diferentes muestras analizadas en cada estudio. También se empleó el software Mass Profinder de Agilent para realizar las etapas de extracción de entidades y su alineamiento en una única etapa.

- Análisis estadístico. Se utilizó el software Statgraphics para realizar distintos análisis estadísticos univariantes y multivariantes, así como para los estudios de superficies de respuesta con los que obtener los valores óptimos de distintas variables. También se empleó el software Mass Profiler Professional, que permite la aplicación de una gran variedad de algoritmos para el análisis estadístico especialmente adecuados para el análisis metabolómico. Algunos de los utilizados en los estudios recogidos en esta Memoria fueron: análisis de varianza (ANOVA) para evaluar compuestos responsables de la diferenciación entre muestras y análisis no supervisado mediante componentes principales (PCA) para detectar agrupamientos y el nivel de diferenciación entre muestras.

5. Bases de datos

Existen varias bases de datos de metabolitos a disposición del usuario que contienen información para la identificación y caracterización de numerosos compuestos presentes en las diversas matrices biológicas. Entre ellas, las bases de datos "Metabolites and Tandem MS Database" (METLIN), "Human Metabolome Database (HMDB), "MassBank" y "PlantCyc" contienen información sobre la naturaleza de los compuestos, así como sobre su ionización y fragmentación en espectrometría de masas; lo que ha permitido la identificación de compuestos presentes en los extractos de piel de naranja, estevia rebaudiana y ajo fresco y negro según se recoge en los Capítulos II, IV, IX y X de esta Memoria. La base de datos "NIST" se ha empleado para la identificación de compuestos presentes en la fracción volátil de ajo fresco y negro (Capítulos VII y VIII). Además, la base de

datos "Kyoto Encyclopedia of Genes and Genomes" (KEGG) se ha utilizado para la interpretación de los resultados y la identificación de rutas metabólicas implicadas en los estudios recogidos en los Capítulos III, IX y X.

PARTE EXPERIMENTAL

PARTE A

Caracterización de residuos agroalimentarios: extracción y análisis de compuestos de alto valor añadido procedentes de la piel de la naranja

Esta primera parte experimental de la Tesis también corresponde a los primeros estudios realizados por la doctoranda. Por tanto, en ella se recoge en primer lugar el fruto de su formación en la literatura existente que sería el soporte de la investigación futura: los aspectos relacionados con el pretratamiento y preparación de las muestras vegetales y los métodos analíticos existentes para determinar propiedades genéricas o específicas de los componentes de las muestras analíticas obtenidas fueron concienzudamente asimilados por la doctoranda. El Capítulo I de esta parte A —y que a su vez constituye un capítulo de un libro multiautor— es un claro exponente del rigor con el que llevó a cabo el estudio crítico de la bibliografía sobre el tema en que versaría su Tesis.

La aportación de la investigación realizada para un mejor conocimiento de un residuo como el proveniente de la industria de zumos de cítricos se recoge en los Capítulos II y III. Este estudio, que pretende aportar información para una explotación exhaustiva de los citados residuos, ha proporcionado la información necesaria sobre una vía de obtención simultánea de extractos polares y apolares de los residuos con el auxilio de ultrasonidos para favorecer la emulsión de los extractantes y la extracción de los compuestos —metabolitos secundarios— para su posterior separación e identificación mediante un equipo constituido por un cromatógrafo de líquidos y un detector de masas de tipo cuadrupolo-tiempo de vuelo (LC–QTOF), según se refleja en el Capítulo II. La siguiente aportación en este campo la constituyó el estudio de la mejor forma de conservación de estos residuos (secados en horno o liofilizados en comparación con la materia fresca) para la obtención de una de las familias de mayor interés entre las que los componen: los flavonoides. La separación y determinación cuantitativa de estos metabolitos se realizó mediante LC–QqQ y puso de manifiesto que las formas glicosiladas se preservan mediante liofilización, mientras que son las agliconas las predominantes en el material sometido a secado en horno, tal como recoge el

Capítulo III.

CAPÍTULO I

Extraction, separation and identification/quantitation of valuable compounds from agrofood materials

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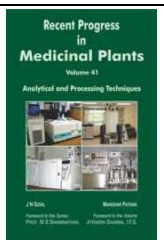

Extraction, separation and identification/quantitation of valuable compounds from agrofood materials

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ABSTRACT

The new extraction techniques assisted by auxiliary energies such as ultrasound or microwaves, or by superheated liquids or supercritical fluids offer key advantages over traditional extraction techniques that are compared in this chapter. The ways for high-resolution individual separation of the extracted compounds —mainly based on liquid chromatography (LC), gas chromatography (GC) or, less commonly, capillary electrophoresis (CE)— coupled to common molecular absorption or emission detectors are used for routine quantitation of known compounds. Identification and accurate quantitation require mass spectrometers (MS), single or in-tandem, with time-of-flight (TOF) capability, etc., giving place to present arrangements in which MS triple quad, Qq/TOF are connected to any of the high resolution separation tools (LC–MS triple quad, LC–Qq/TOF, GC–MS/MS ion trap, GC–Qq/TOF, etc.). This equipment also allows detection, identification and quantitation of the degradation products, sometimes of higher value than their precursors.

Keywords High-value compounds; extraction; auxiliary energies; sample profiling; targeted analysis; mass spectrometry.

1. Introduction

Agrofood is one of the main industrial sectors over the world, as it produced about 132 millions of tons of food in the European Union (EU) in 2012 [1]. Most of these industries focus their activity on obtaining a single high-value product, generating during the process other products with low or non value; that is, residues or wastes. A pending goal in this sector is to achieve an integral exploitation of residues and wastes, to which the present call for proposal of the EU (Waste7 under the umbrella of Horizont 2020 [2]) contributes.

Key aspects to be covered as a premise to reach the pursued goal are improvement of the processes to extract target compounds from given residues and development of analytical methods based on cutting-edge technology for identifying the components, then performing individual separation, if required.

Simple and complex methods and their scope are discussed together with examples of their use in order to show the field of application, advantages and limitations of them, thus opening a door to their correct use and the possibility of innovating for a better achievement of the pursued objective.

2. Extraction methods

Extraction is a sample preparation step critical for the success of the analytical process since its effectiveness strongly influences the quality of the final results. The extraction step is usually aimed at fulfilling four main objectives: (i) to separate the compounds of interest from the sample matrix; (ii) to remove interferents that could affect measurement of the target compounds; (iii) to transform the sample into an extract compatible with the analytical equipment; and (iv) to concentrate trace analytes before analysis, if necessary [3].

The selection of the extraction method depends on both the compounds to be

extracted and the matrix from which they should be removed. The physical state of the sample allows classification of extraction into solid–liquid extraction (more properly known as leaching or lixiviation) when the sample is a solid, while liquid samples can be extracted to an immiscible liquid —giving place to liquid–liquid extraction (LLE)—, or to a solid in normal format (solid-phase extraction —SPE) or micro format (solid-phase microextraction —SPME) [4].

The extraction of valuable compounds from agrofood materials have traditionally been performed using conventional maceration or Soxhlet methods; all them requiring simple equipment, long extraction times and large volumes of extractants. The two last negative aspects (crucial in routine extraction and in fulfilment of environmental-friendly policies, respectively) have promoted ways for minimizing/eliminating them by developing methods that shorten the extraction time, decrease the extractant volume and automate the process in a partial or total manner.

Several have been the ways to achieve the aims that, in short, modernize the old methods: (i) application of auxiliary energies, such as microwaves and/or ultrasound, giving place to microwave-assisted extraction and ultrasound-assisted extraction (MAE and USAE, respectively); (ii) use of superheated liquids or supercritical fluids (giving place to superheated liquid extraction and supercritical fluid extraction —SHLE and SFE, respectively) both also involving in some aspects auxiliary energies, such as those required to increase the temperature and/or pressure.

2.1 Use of auxiliary energies to favor the extraction process

One of the most common auxiliary energy to favor extraction processes is that provided by microwaves (MW). MAE is highly dependent on the physical and chemical characteristics of the extractant as the mechanisms of interaction with it are a function of both the dielectric constant and polarity of the target

system [5]. MW have also the advantage of destroying the sample matrix because their capability to break the cells, thus favoring release of the target compounds [6, 7, 8].

Two main types of MW extractors can be distinguished depending on the random or focused mode in which this energy is applied to the sample [9]. Other classifications of these devices can be established as a function of their working mode (batch or continuous), examples of which can be found elsewhere [9], or the types of vessels and pressure inside them (closed or open systems). Closed extractors are commercialized by CEM or Milestone, while open extractors are mainly custom-made (sometimes from a household oven).

MAE provides fast extraction methods as compared to other alternatives as SHLE. Thus, the time for extraction of antioxidants from *Folium eriobotryae* is reduced from several h in the case of SHLE to 3 min for MAE [10] and for the extraction of oleuropein and related phenols from olive leaves, this time is reduced from 24 h in case of maceration at 40 ^{o}C to 8 min for MAE [11]. Regarding acquisition costs of the required equipment, MW extractors are cheaper than those for SHLE or SFE. Nevertheless, MAE has negative aspects such as low selectivity, and extraction heavily dependent on the nature and temperature of the solvent [12]. Moreover, MW application causes the increase of temperature, which could affect to thermolabile compounds and generate degradation products. Alternative devices to carry out MAE have been developed with the purpose of decreasing or overcoming these drawbacks, as is the case with the vacuum MAE, aimed at reducing both degradation of thermolabile compounds and oxidation processes [13]. The comparison of the performance of such a devices with a typical MW extractor to remove vitamins C and E from guava, green pepper, soybean and tea leaves showed that vacuum MAE increased the yield of the vitamins in 20–145%, depending on the selected sample.

USAE has as advantages over MAE and other extraction techniques of having

no restrictions concerning the extractant to be used and the negligible change of the extractant temperature, which allows extraction of thermolabile compounds without degradation. Most of the research carried out by USAE has so far used an US cleaning bath. It is a cheap and accessible device, omnipresent in any laboratory, that is affected by a lack of uniformity in the transmission of US and a decline of power with time, so this device has a low reproducibility in extraction, a function for which this device has not been designed and commercialized [14]. There are at present in the market different US baths specifically designed for chemical purposes (synthesis, extraction, etc.), in addition to the well-known US probes that work either by direct immersion into the sample–extractant system or through a water bath where both the container with the sample–extractant system and the probe are immersed. Specific baths and probes are more expensive than cleaning baths, but they provide more reproducible results and allow optimization of sonication parameters such as amplitude and duty cycle of US, and even US tuning frequency in some devices [14, 15].

As in the case of MW, US causes disaggregation and rupture of the cell walls, promoting the release of target compounds outside the cells [15]. The cavitation phenomenon characteristic of power US (name of US in the range 20–40 kHz) allows an effective contact between the extractant and the sample, and also erodes the solid surface, both phenomena enhancing mass transfer [16, 17]. The effects of US to reduce the extraction time as compared with Soxhlet extraction are shown by the extraction of oil from grape seeds: 30 min are required for the former method *versus* 6 h needed by conventional Soxhlet. The cavitation effect also promotes a higher extraction yield, as shown by comparison of USAE, Soxhlet extraction, SFE or MAE to extract of oil from pomegranate seeds (yields 56.8, 18.7, 3.21, 15.8%, respectively) [18, 19].

US not only facilitates solid–liquid extraction by a single extractant with affinity for compounds of given chemical characteristics, but also two immiscible

extractants can be used to extract compounds of very different nature. The fact that cavitation favors emulsion of the two extractants drastically increases the liquid surface that, together with erosion, accelerate mass transfer to both liquid phases, which can be easily separated by decantation or centrifugation into the two extracts that contain polar and non-polar compounds obtained by a single extraction step [20]. The addition of a derivatizing agent (if necessary for subsequent identification and/or quantitation) to one or the two extractants also favors displacement of the mass transfer equilibrium [21].

In dealing with liquid samples, US also favors liquid–liquid extraction by formation of emulsions that increase the contact surface, and the mass transfer as a result [22].

One negative aspect of cavitation, particularly in polar media, is the formation of free radicals that can induce degradation via hydrolysis and oxidation, among other reactions; thus affecting the extraction yield of compounds sensitive to degradation [14]. An in-depth study is mandatory in this case as the products resulting from degradation can be either toxic, undesirable, or of greater interest than the parent compounds.

2.2 Use of superheated liquids and supercritical fluids

SHLE uses a liquid heated above its boiling point, but subjected to enough pressure to keep under the liquid state. There has been much debate over the name this technique should be given ever since its inception. Dionex Corporation initially patented it under the designation "accelerated solvent extraction" (ASE) [23], which was also applied to its commercial devices. With time, however, alternative names such as "pressurized fluid extraction" (PFE), "pressurized hot solvent extraction" (PHSE), "high-pressure solvent extraction" (HPSE), "subcritical solvent extraction" (SSE), "subcritical water extraction" [24], "superheated solvent extraction" (SHSE) and "superheated liquid extraction"

(SHLE), among others, have gradually replaced ASE. In the authors' opinion, high pressure is not the most salient feature here since, most often, the only purpose of raising the pressure is to keep the extractant in the liquid state and only in a very few cases has an increase in pressure above this level had any effect. On the other hand, the term "subcritical solvent" encompasses any temperature and pressure below the critical point, even at ambient conditions, and is, therefore, inappropriate as well.

SHLE can be done under static conditions, dynamic conditions —by continuously circulating the solvent through the sample— or a combination of both. The variables affecting performance in SHLE include temperature; pressure; type, volume and —if a dynamic mode is used— flow-rate of extractant; matrix composition; sample size and extraction time. Most solvents can be used as SHL extractants, excluding those with auto-ignition temperatures within 40–200 ºC (*e. g.* diethyl ether, 1,4-dioxane) [25]. The state of these extractants provides a high extraction efficiency; at short times and with low extractant volumes.

Comparison of SHLE–MAE–Soxhlet methods has been made on samples with analytes very difficult to extract, as is the case with nitrated polycyclic aromatic hydrocarbons (nitro-PAHs) extracted from soils and requiring times of 11 min, 20 min and 15 h, respectively [26]. Therefore, less retained analytes, as those naturally existing in plants, require very short extraction times —in the order of few min— for quantitative removal.

Supercritical fluids —SF— (those at temperature and pressure above their critical point) had a brilliant time both as extractants and mobile phases in chromatography (SFC) by the 1990 decade. Supercritical CO_2 is the solvent most used for the extraction of compounds, although methanol, water, ammonia or *n*-hexane have sporadically been used [27]. The success of CO_2 in SFE is attributed to its high purity, low critical temperature and pressure and low toxicity; however, CO_2 also has as limitation to be non-polar, thus, it only can extract compounds

with medium or non- polarity and requires the use of modifiers —polar co-extractants— for more polar compounds.

Problems related to different behavior of spiked and naturally existing analytes in SFE, and to irreproducible behavior as mobile phase in SFC relegated both to a minimum use. Present better understanding of matrix effects in SFE have delimited its use in the food field, mainly for fat extraction prior to determination; while improvements in mobile phase delivery, reproducible modifier introduction and greater analytical sensitivity SFC occurred within the last few years have created a position to this type of chromatography [28]. Nevertheless, the high price of enough pure CO_2 makes SFE only amenable to obtain high-priced compounds such as flavors and fragances from plants, both at the laboratory and industrial scales [29]; and established industrial processes such as deterpenation of orange peel oil [30] and decaffeination of coffee beans.

3. Analysis of the extracts

Subsequent manipulation of the extracts depends on the final purpose pursued by manipulation of the initial raw material, which can be: (i) to obtain a single, target compound, thus making mandatory a generally laborious and time consuming process of separation and purification involving, most times, a number of steps. This is a preparative purpose, while the others are analytical in nature, as schematized in Fig. 1. (ii) To know a given property of the raw material, which can be studied through the obtained extract (*e.g.,* antioxidant capacity, total polyphenols index —TPI— radical scavenging, FRAP, ORAC or TEAC). This information is most times obtained by a single analysis —which can be a simple step as is the case with the antioxidant capacity by the TPI or the Folin–Ciocalteu method; or a complex single step as is the case with the ORAC test. (iii) To identify the polar/non polar compounds (depending on the polar/non polar extractant

used to obtain the extract) existing in the raw material. This is a more complex process as it requires expensive equipment such as time-of-flight (TOF) analyzers to obtain the data that then are treated to look for compounds identification in given databases. (iv) To quantify in the extract one single compound or a number of compounds, usually of the same family or with some common chemical characteristics. Obtainment of this information requires appropriate separation of the target compounds using high-resolution equipment such as a liquid or gas chromatograph, or a capillary electrophoresis system —depending on the chemical characteristics of the analytes—, and the use of the corresponding standards, that should be labeled stable isotopic standards if a mass spectrometer is used as detector.

As most of the compounds extracted from agrofood materials are metabolites (mainly secondary metabolites), purposes (iii) and (iv) are identified with profiling/untargeted analysis and with targeted analysis strategies, respectively, commonly used in metabolomics.

Figure 1. Analytical strategies and their tools for the analysis of agrofood materials.

3.1. Isolation/purification of a single compound

Certain well-known compounds with recognized properties are of interest for different industrial fields (pharmaceutical, cosmetics, etc.) where their use requires exhaustive separation from other components of the given extract; therefore, their isolation and purification are key steps for this purpose. These exhaustively developed steps are mainly under patent to be exclusively used by given industries that acquire the rights for exploitation. Occasionally, developments at a laboratory scale are published in scientific journals. A case in point is hydroxytyrosol, which is extracted from either olive oil wastes or from olive leaves, and whose healthy properties have been recognized by the EFSA (European Food Safety Authority) [31]. One of the methods reported for isolation of hydroxytyrosol involves the use of a strong anion exchanger resin loaded in a column to retain the target phenol and waste most of the compounds in the extract (mainly low- or medium-polar olive-waste components); then, hydroxytyrosol is eluted with water for subsequent passage through a polymeric resin for purification purposes, and eluted with an ethanol–water mixture [32]. Other alternative method for isolation/purification of hydroxytyrosol is based on counter-current extraction, an iterative process based on successive automated extractions until obtaining a pure solution of hydroxytyrosol, which requires long times and high volumes of solvents [33].

3.2. Analysis of a given property in the extract

The growing interest in the health benefits of agrofood compounds is mainly due to some specific properties attributed to given components present in given raw material, such as antioxidant and/or radical scavenging capacity or overall content of given families of compounds with recognized properties, such as those attributed to phenols or anthocyans. To determine one or several of these properties, there are simple and rapid well-established analytical methods, either official or standard. Most of them are based on the changes in the absorbance of

the target solution with or without addition of an unspecific reagent. They are characterized by a low selectivity as a number of compounds contribute to the monitored signal in a proportion that depends on their chemical characteristics.

Two of the simplest methods are the total phenols index (TPI) and total anthocyans index (TAI). The former measures the phenols content by monitoring the absorbance of the target solution at 280 nm; therefore, sample dilution, if required, is the only preparation step. On the other hand, the TAI requires acidification of the extract, division into two aliquots, and addition of a solution of potassium pirosulphite to one of them to convert anthocyans into colorless products. The TAI is given by the difference between the absorbance at 520 nm of the extract without and with potassium pirosulphite. The simplicity of this assays allows automation by a flow injection approach [34].

The Folin–Ciocalteu (F–C) reagent gives place to the method known by the name of these two scientists, which is one of the most used for determination of total phenolic antioxidants. The reagent, composed by phosphomolybdate and phosphotungstate, is reduced by antioxidants, producing blue-colored compounds that absorb at 765 nm [35]. The content in phenolic antioxidants is related to the difference in absorbance of the treated extract and a blank. This value is related to the total concentration of phenols by using a calibration curve, run with a standard phenol, and within which the found value is interpolated (galic or caffeic acids are the most common standards used with this aim). This assay is commonly accepted as a measure of the antioxidant capacity of a sample thanks to its advantages: the reagent is commercially available, and the long-wavelength for monitoring minimizes interferences [36]. However, the F–C reagent is sensitive to any reducing compound present in the extract and it needs time and heat to complete the reaction.

The Ferric Reducing Antioxidant Power (FRAP) test is also widely used for the determination of the antioxidant capacity. It is based on reduction of the ferric

tripyridyltriazine [Fe(III)-TPTZ] complex to ferrrous tripyridyltriazine [Fe(II)-TPTZ] at low pH by the presence of antioxidants. The reduced complex has an intense blue color that can be monitored at 593 nm [37]. As in the F–C method, FRAP requires a calibration curve (in this case TROLOX —an analogue of vitamin E— is used as standard) and the reagent is sensitive to any reducing substance present in the sample.

The Oxygen Radical Absorbance Capacity (ORAC) test is also used to measure the antioxidant capacity. The ORAC test monitors the oxidative degradation of fluorescein in the presence of free radical generators such as azo-initiators, but antioxidant compounds protect fluorescein from degradation. The difference in fluorescence of the solution before and after addition of a free radical generator is directly related to the concentration of antioxidants, which is quantified by a calibration curve using TROLOX as standard. The drawbacks of this assay is that a fluorimeter is required (less common in a laboratory and with a price higher than that of a photometer) and that about 35 min are required to complete the assay.

A similar assay is TROLOX Equivalent Antioxidant Capacity (TEAC), where the radical anion of 2,2'-azinobis-(3-ethylbezthiazoline-6-sulfonate) (ABTS) is used to predict the antioxidant capacity of the extract. In it, an ABTS solution is mixed with the extract and the decrease in absorbance at 734 nm before and after mixing is directly related to the concentration of antioxidants. The antioxidant power is expressed as TROLOX capacity. TEAC is a fast assay and the antioxidants in the sample are discriminated as those that have an immediate action and those that needs time of action, by monitoring the absorbance immediately after mixing sample and reagent, and after 6 min [38]. However, ABTS requires previous steps to obtain the radical form, which have a short lifetime.

The most used radical scavenger is 2,2-diphenyl-1-picrylhydrazyl (DPPH) as it is a stable free radical with a deep violet color that, when neutralized by other

radical present in the extract, turns into non-colored or pale yellow product. This assay measures the loss of absorbance at 520 nm from free-radical DPPH solution to the neutralized DPPH solution when in the presence of the extract (neutralized DPPH). The reaction is slow, as the assay needs a stabilization time of 15 min, approximately [39]. The result is given as percentage of inhibitor action to DPPH.

3.3. Identification and quantitation of compounds in the extract

This section encompasses points (iii), untargeted or profiling analysis; and (iv), targeted analysis.

(iii) Untargeted or profiling analysis

Untargeted analysis is focused on identification of all analytes or most of the components in the extract, with relative or absolute quantitation depending on the absence or presence of the corresponding standards. The data set obtained by a given equipment is very extensive, so it requires treatment with advanced chemometric techniques to be turn into a manageable signal set. With this aim, different procedures can be followed, such as identification by using standards or the comparison data set provided by a potential compound with specialized databases (as is the case with MS analysis). Hence, untargeted analysis gives the opportunity to identify new metabolites with a specific function inside the metabolome of the plant material, which can be endowed with exploitable properties. This strategy has a number of limitations: processing of raw data sets requires long times (weeks or months), the identification of unknown compounds represents a challenge sometimes hard to overcome, and the detection of highly abundant metabolites can mask the lower signal provided by minor compounds [40].

Analytical tools for untargeted analysis

Untargeted analysis aims to detect in the sample as much compounds as possible, and this set of compounds can be characterized by an enormous chemical

and structural diversity. Therefore, there is a necessity for versatile analytical techniques with high sensitivity and resolution. Nuclear magnetic resonance (NMR) spectroscopy and MS are frequently used as detection techniques in untargeted analysis.

NMR spectroscopy is particularly appropriate for the analysis of bulk compounds and it is suitable for profiling analysis. This is a highly reproducible technique with the advantages of being non-destructive and requiring rapid and simple sample preparation (even it can be used for direct analysis under given conditions). A previous chromatographic separation is most times not required; however, this fact involves as main shortcoming the difficult to separate the signal provided by different compounds, especially in complex samples, as is the case with plant extracts, which generates a large number of peaks in a small chemical shift range, especially in ^{1}H-NMR spectra. Therefore, peaks generated by different compounds have more chance of overlapping with each other and, as a result, potentially key compounds present at smaller concentrations are often overshadowed by larger peaks [41].

MS detectors constitute excellent tools for untargeted analysis, whose resolution can be improved by hyphenation to high-resolution separation equipment. Although direct injection mass spectrometry (DIMS) provides a rapid fingerprint and it has demonstrated to be valuable for a first-round screening of material [42], the combination of MS and a previous separation technique has gained importance in the field of agrofood-material analysis.

No any type of mass detector is appropriate for untargeted analysis as not all them has enough capability for identification. One of the most used in this field is the quadrupole-time-of-flight (QTOF) mass spectrometer, as it is able to ionize the compounds (MS mode) providing an appropriate fragmentation and high mass accuracy, particularly in the MS/MS mode, which generate a large amount of information for subsequent identification. This mass analyzer is endowed with a

high selectivity, meanwhile sensitivity is lower than in other mass analyzers such as the triple-quadrupole mass spectrometer (QqQ). To increase the identification capacity by separation of compounds providing similar fragments, many researchers use QTOF spectrometers coupled to either GC, LC or even CE equipment for untargeted and fingerprinting analysis. One of these couplings (LC–TOF/MS in this case) was used to discriminate among refined edible oils enriched with phenolic extracts from olive leaves and from "alperujo". Distinction among the enrichment degree in each phenol as a function of fatty-acid composition of oils and the relative concentration of phenols in the extracts was achieved, then explained through the chemical features of the phenols/fatty acids binomial [43]. Comparison of the use of GC–MS and two-dimensional GC–QTOF (GC×GC–TOF) to analyze essential oils from leaves of *Polygonum minus* clearly demonstrated the higher resolution of the latter (about five times more identified compounds than those reported by GC–MS) [44].

One other mass detector used for untargeted analysis has been that based on ion trap, which has frequently been hyphenated to GC, thus focused on the analysis of volatile compounds or easily converted into volatiles by derivatization. As an example of the latter is the research by Mijangos-Ricárdez *et al.*, who analyzed the lipidic fraction of arnica, a Mexican traditional medicine, by GC–MS ion trap, after conversion of the fatty acids into their methyl esters [45].

(iv) Quantitation of target components in the extract

Targeted analysis is based on the measurement of known and well characterized groups of compounds within a plant material. Absolute quantification is carried out by comparison with standards of the target compounds that not always are commercially available. In the absence of these key standards, target components can be relatively quantitated by using the calibration curve of the compound more similar for which commercial standard exists.

By quantification of specific key metabolites with well-defined functions in metabolic pathways, perturbation in these pathways can be detected, which can provide information about the state of the target plant, such as state of maturation [46] or resistance against herbicides [47], which can be subsequently used to improve the quality of the crop for a given purpose. Targeted analysis, in contrast with its untargeted counterpart, is focused on the analysis of a reduced group of compounds; therefore, it requires the removal of potential interferents, modifying the extraction step or including additional separation steps, such as solid-phase extraction (SPE) that can be coupled on-line with the individual separation–detection steps.

Analytical tools in targeted strategy

Quantitation of given compounds characteristic of targeted analysis requires an effective removal of potential interferents; therefore, sample preparation is usually more exhaustive and longer than for untargeted analysis. Additionally, a preconcentration step can be required either because the analytes are low concentrated in the original sample or because dilution occurs along the steps involved in sample preparation.

SPE is one of the most common and useful tools for cleanup and concentration before analysis of the compounds of interest. There are two general procedures depending on the sorbent material packed in the cartridge: (i) the usual mode, which consists of retention of the target compounds in the sorbent and, after the rest of the extract components have been wasted, the sorbed compounds are eluted with the minimum volume of the eluant. (ii) The target analytes are not retained by the sorbent and pass through it, while the interferents remain absorbed in it. Generally, after collecting the analytes, the interferents are eluted with the proper solvent; thus, the cartridges can be reused. One advantage of SPE is on-line coupling to the analytical tool, allowing an effective transfer of the compounds without losses, and automated performance of the overall process [48].

A characteristic device for on-line sample preparation prior to GC individual separation of volatile species is head space (HS), in which the sample is heated in a hermetically closed vial and the atmosphere created over the sample level that contain the volatilized compounds is totally or partially injected into the chromatograph [49]. In headspace solid-phase microextraction (HS–SPME) the volatile target compounds are retained in a fiber in contact with the HS atmosphere and, subsequently, the volatile compounds are released by thermal desorption [50].

On the other hand, separation of less volatile compounds by GC requires a derivatization reaction. This sample preparation step was applied prior to the analysis profile of sugars in olive fruits by GC–MS/MS. The derivatizing reagent *N,O*-bis(trimethylsilyl)trifluoroaceteamide (BSTFA) was used, by which the extracted sugars were converted into their trimethylsilyl derivatives [51].

Apart from the well-established use of GC for individual separation of volatile compounds or derivatization products such as essential oils or fatty-acid methyl esters, respectively, most of the target compounds found in an extract can be separated by LC. The different formats of a liquid chromatograph (from conventional LC to that working in ultra-performance —UPLC) allow fulfilling all possible necessities concerning sample size and separation velocity of the given compounds. Other variables to be taken into account for obtaining optimum results are related to the nature of the mobile phases and the stationary solid-phase packed in the columns giving place to reversed-phase columns (the most used are silica-C_{18} based columns), with strong interaction with low-polarity compounds [52]; and normal-phase columns (such as HILIC columns endowed with amino groups for interaction), with better interaction with high-polar compounds [53]. An on-line combination of two columns (a C_{18} and an amino columns) has recently been proposed for the analysis of stevia extracts [54].

Also capillary electrophoresis (CE) is a separation technique widely used in

targeted analysis. Both aqueous and not aqueous electrolyte backgrounds have been used to promote the required separation of the target analytes. Thus, an aqueous solution as buffer has been used to separate permanently charged compounds such as anthocyans, quaternary alkaloids and sulphated flavonoids [55], wherein the pH of the buffer solution can be varied without losing electrophoretic mobility. Other compounds that possess phenolic hydroxyl groups requires a neutral to basic pH, working in the range 7–12 and those that cannot be classified into acids or bases (*e.g.*, sugars and phenolic glycosides) require a previous derivatization step [56]. This last is the case of reduced and derivatized sugars to their *N*-2-pyridylglycamines, then complexed with borate to reach a proper electrophoretic mobility. On the other hand, non-aqueous buffer systems based on water-miscible organic solvents such as acetonitrile (ACN), dimethyl formamide and alcohols together with formic acid, acetic acid, ammonia or diethylamine have been used to favor the solubility of less-polar or non-polar compounds [57].

Concerning detection in targeted analysis, very different types of detectors have been used depending on the compounds to be quantified. Spectrophotometric detectors, such as conventional UV/visible spectrophotometers or diode-array detectors (DAD), and fluorescence detectors (FLD), with a conventional or laser excitation source, have been widely used because of their facility of use, their relatively low cost and robustness. Many compounds absorb visible or UV radiation; however, few of them exhibit intrinsic fluorescence. This is the reason why fluorescence is most times promoted by a derivatizing reagent. As example, Li *et al.* derivatized free fatty acids from *Swertia* species with 2-(5-benzoacridine)ethyl-*p*-toluenesulfonate prior to their individual separation by LC and FLD detection [58].

Flame ionization detector (FID), coupled to GC, is one of the most used detectors for analysis of volatile compounds with intrinsic volatility or acquired

by product formation. As compared to MS detectors, FID is an easy-to-use and cheap detector, characterized by high sensitivity, but without capacity for identification.

MS-based detectors are the most versatile and with the highest capacity for quantitation, with also possibility for identification. One of the most sensitive MS detectors at present is the triple-quadrupole mass spectrometer (QqQ), in which the compounds are ionized and fragmented, and the detector provides a signal in the so-called *selected reaction monitoring (SRM)* mode corresponding to a transition from precursor ion to product ion. Due to the usual multiple fragmentation of the compounds, an optimization of the ionization and fragmentation parameters is required to select the fragment with the highest signal without complete loss of the precursor ion. The transition thus obtained is used for quantitation by interpolation within the linear portion of the calibration curve obtained with standards of the given compound. An example of the use of LC–QqQ is for targeted analysis of triterpenoid compounds after extraction from olive leaves [59]. After exhaustive optimization of the variables affecting detection, on column limits of detection within the range 0.26 to 0.91 ng were obtained.

4. Trends

The most ambitious trend in taking benefits from agrofood residues and wastes is integral exploitation, towards which governments of most of the developed countries channel their endeavors. The efforts in this case are focused on development of special programs such as the above commented Waste7 [2], or in the case of US, the efforts are oriented to either programs to diversify products as in the case of cellulose through pushing research on nanocellulose transformation [60, 61], or concrete research on a given waste such as tomato skin, waste massively generated in the production of ketchup, the use of which revealed

as a reinforcing component for plastic composite car parts such as wiring brackets and coin storage bins [62].

These research guidelines will lead undoubtedly to new applications of the present residues and wastes, thus involving higher benefits from a given crop and the corresponding decrease of disposal in landfills as a result.

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CAPÍTULO II

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Ultrasound-assisted emulsification–extraction of orange peel metabolites prior to tentative identification by LC–QTOF MS/MS

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Ultrasound-assisted emulsification–extraction of orange peel metabolites prior to tentative identification by LC–QTOF MS/MS

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ABSTRACT

An optimized method for extraction and characterization of compounds present in orange peel has been developed. The extraction method allows the simultaneous extraction of polar a non-polar compounds by using two immiscible extractants (a polar extractant —an 80:20 (v/v) methanol−water mixture—, and a non-polar extractant —*n*-hexane). Optimization of the ultrasound-assisted emulsification–extraction (USAEE) led to the following values as desirability conditions for both extracts: 32% amplitude, 0.75 s/s duty cycle and 7.5 min of extraction time. The extracts obtained under these conditions were analyzed by LC−QTOF MS/MS in positive and negative ionization modes. Tentative identification of the most significant compounds present in each extract allowed their characterization by using high resolution tandem mass spectrometry. The optimum extracts provided by USAEE were compared by using Principal Component Analysis to those obtained by conventional extraction based on maceration. Thus, the composition of the polar extracts obtained after 7.5 min ultrasonication was similar to that of conventional maceration for 4 h in both ionization modes. On the contrary, the analysis of non-polar extracts led to different results depending on the ionization mode: the ultrasound-assisted extract was similar to those of conventional maceration for 7 min and 10 h in negative and positive ionization, respectively. This behavior could be explained by the contribution of different groups of compounds to each ionization mode.

Keywords Metabolic profile; orange peel; ultrasound-assisted emulsification–extraction; LC–QTOF analysis; industrial wastes

1. Introduction

Orange is the largest fruit crop worldwide with more than 60% of the total citrus crops [1]. Approximately 40% of the orange production is devoted to elaboration of orange juice, which generates residues of 50–60% of the total raw material [2]. These residues —formed by peel, which is constituted by flavedo and albedo (non-edible parts of the fruit), and seeds— have traditionally been transformed into pelletized bran for animal feeds [3]. Despite the number of interesting compounds this residue contains, its in-depth exploitation has so far not been achieved. In widely spread crops such as olive trees and vineyards, a comprehensive use of the residues is carried out to obtain secondary high-valued products [4]. The majority of the studies related with orange peel has been focused on tentative identification of particular families in these residues —mainly metabolites with a high interest for the pharmaceutical, cosmetics, food or nutraceutical industries— and the development of successful extraction methods for target families [5].

Key metabolites present in orange peel are flavonoids, a group of phenolic compounds with nutraceutical benefits related to their antioxidant activity and radical scavenging capability. Thus, numerous studies have proved the anti-inflammatory and antimicrobial activity of flavonoids [6,7], as well as their prevention role against atherosclerosis and cancer [8–11]. Among flavonoids present in orange peel, it is worth mentioning aglycon forms such as hesperetin, naringenin and apigenin, and glycoside forms, such as neohesperidin, hesperidin and naringin [11]. One other important family of compounds in orange peel is that constituted by essential oils: non-polar metabolites such as terpenes, fatty acids, carotenoids and other volatile compounds, being limonene the most representative and abundant among them [12]. Orange essential oils have been used as aroma flavor in many foods such as beverages, marmalades, sweets and candies [13,14], and also as head notes in cosmetics and perfumes [15]. Additionally, biological studies have attributed antibacterial [16], antimicrobial [17], and insecticidal activity [18] to these essential oils.

Different extraction methods have been tested for isolation of natural products from orange peel. The first approaches were based on conventional methods such as Soxhlet extraction [19], maceration [20], steam distillation [21] or cold pressing [22]. However, these methods need long extraction times as in the study from Guimarães *et al.*, who extracted polar compounds from different citrus peel and juice by maceration for 12 h and extracted volatile compounds from the same matrices by hydrodistillation for 3 h [23]. Moreover, these methods have led to incomplete extraction, as proved Xu *et al.*, who used maceration with hot water for the extraction of flavonoids from orange peel and achieved extraction yields below 55% regarding to the total content in the orange peel [24]. Later, supercritical fluid extraction (SFE) was proposed for the extraction of citrus essential oils using supercritical CO_2 [25] or ethane as extractant alternative [26]. Nowadays, new extraction methods based on the use of auxiliary energies are being developed. Application of microwaves (MW) or ultrasound (US) allows rapid and cheap extraction methods that shorten the extraction time and lower extractant consumption [27,28]. As example, Khan *et al.* optimized an US-assisted extraction method for isolation of phenolic compounds, although they only determined the total phenolic content and the antioxidant capacity of the extracts. Identification was restricted to two flavonoids, hesperidin and naringin, which were used as behavioral model of flavonoids [29].

Simultaneous extraction of polar and non-polar compounds can be achieved with the help of US by the approach known as US-assisted emulsification–extraction (USAEE) that has proved to be very efficient [30]. It is based on the use of two immiscible extractants, which, under ultrasonication, form an emulsion that allows close contact of both extractants with the solid sample and a rapid mass transfer of the compounds extractable to one or the other liquid phase with the help of the cavitation phenomenon, also promoted by US. Thus, USAEE is an effective extraction method for characterization of the metabolic profile of a given solid sample. In the case of liquid samples, the process consists of liquid–liquid extraction in which US-assisted emulsion

involves the sample and an immiscible extractant (usually a non-polar extractant as the sample is most times aqueous, such as contaminated water, urine or saliva) [31,32].

The excellent performance of USAEE with vegetable matrices led us to develop a method for extraction of as many components of orange peel as possible with a view on optimizing resources and shortening extraction processes. With this final aim, the objectives of the present research were: (i) to optimize the extraction of polar and non-polar compounds present in orange peel using USAEE and demonstrate its efficiency; and (ii) to identify the most representative families of metabolites present in orange peel.

2. Materials and methods

2.1. Samples and reagents

Edible oranges (*Citrus sinensis)* were purchased in a local supermarket, then squeezed and the peel was lyophilized. The resulting solid was grinded in a cyclone mill. All solvents were LC grade or higher. Deionized water (18 MΩ·cm) was from a Millipore (Bedfore, MA, USA) Milli-Q plus system. Methanol from Panreac (Barcelona, Spain), *n*-hexane from Scharlab (Barcelona, Spain) and acetonitrile (ACN) from Fluka (Buches, Switzerland) were also used.

2.2. Apparatus

After freezing at –80 ºC for 24 h, the orange peels were lyophilized in an FTS Systems lyophilizer (New York, United States). Subsequently, all the dried material was grinded in a cyclone mill (Tecator Cyclotec1093 Sample Mill) to a particle size below 500 μm.

Ultrasound was applied by a Branson 450 digital sonifier (20 kHz, 450 W) equipped with a cylindrical titanium-alloy probe (12.70 mm diameter). A magnetic stirrer and a centrifuge (Selecta, Barcelona, Spain) working at 2200 *g* for 10 min were

used. The non-polar extract was concentrated in an Eppendorf Concentrator 5301.

An Agilent 1100 liquid chromatograph (LC) connected to a Diode Array Detector (DAD) was used to analyze the extracts during the optimization step. The LC system was furnished with a Rheodyne 7725 manual injection valve and a 20 µL injection loop. The analytical column was an Inertsil ODS-2 (5 µm, 4.6 × 250 mm) from GL Sciences (Tokyo, Japan).

An Agilent 1200 LC coupled to a 6540 UHD Accurate-MS QqTOF was used to identify the metabolites in the extracts. The analytical column (Inertsil ODS-2) used in LC–DAD analysis was also used for LC–MS/MS characterization.

2.3. Ultrasound-assisted emulsification–extraction

The assembly for this type of extraction was previously described by Pérez-Serradilla *et al.* [30]. In the present study, 1 g of milled orange peel was placed in an 100-mL beaker with 30 mL of non-polar extractant (*n*-hexane) and 25 mL of polar extractant (a 80:20 (v/v) methanol–water mixture). The US probe was directly dipped into the baker at 0.5 cm deep. The US working conditions were output amplitude 32% of the converter applied power (450 W), and duty cycle of 0.75 s/s. Ultrasonic energy was applied for 7.5 min. Subsequently, the emulsions were disrupted by centrifugation at 2200 *g* for 10 min and the polar and non-polar extracts were separated. The polar extract —which also contained the solid residue— was filtered by a 0.20 µm filter and both extracts were stored at –20 ^{o}C until use.

2.4. Experimental design

The study of the extraction process was supported on a Box-Behnken design by application of response surface methodology. The design evaluated 3 levels for each studied factor, namely US amplitude (X_1), duty cycle (X_2) and extraction time (X_3). The resulting experimental design included fifteen experiments by defining the minimum, maximum and average values for each parameter: 20, 35 and 50% of US amplitude; 0.5, 0.7 and 0.9 s/s of duty cycle; and 2, 5 and 8 min of extraction time. The extracts were

analyzed by LC–DAD using as response variables (Y_k (x)) the three most intense peaks within each of the two peaks groups observed in the chromatograms provided by analysis of the polar and non-polar extracts. The experimental data were fitted into a second order polynomial model with the following equation:

$$Y_i = \beta_0 + \beta_m X_m + \beta_n X_n + \beta_{mm} X_m^2 + \beta_{mn} X_m X_n + \beta_{nn} X_n^2$$

A desirability function approach was used to select the condition for each factor that resulted in the best extraction for both extracts according to the following equation:

$$D = (d_1(Y_1) d_2(Y_2) \dots d_n(Y_n))^{1/n}$$

being d_i (Y_i) a desirability function for each response Y_i (x). The d_i (Y_i) function is equal to 1 representing a completely desirable or, in this case, the ideal response value, obtained by the surface response model. The results were processed using the software Statgraphics Centurion XVI (StatPoint Technologies 2011, USA).

2.5. Conventional extraction

The process was similar to USAEE, but in the absence of US to know the effect of this energy on the process; therefore, 1 g of milled orange peel was placed in an 100-mL beaker with 30 mL of non-polar extractant and 25 mL of polar extractant; then, the vial was closed to avoid losses by evaporation. The mixture was magnetically stirred to favor mass transfer to the liquid phases. Four extraction times were studied: 7 and 30 min, 4 and 10 h.

2.6. LC–DAD analysis

The non-polar extracts were evaporated and reconstituted in 100 µL of *n*-hexane prior to LC–DAD analysis; while the polar extracts were 1:30 diluted with polar extractant prior to LC–DAD. The mobile phases used were deionized water (A) and ACN (B). The constant flow rate was 1 mL/min and the injection volume was 20 µL. The gradient for analysis of the non-polar extract was as follows: 30% B until min 2; change from 30% to 60% B in 15 min; from 60% to 100% B in 12 min and constant 100%

B for 15 min. For analysis of the polar extract the gradient was 4% B until min 10; change from 4% to 20% B in 10 min, from 20% to 50% B in 15 min, from 50% to 80% B in 10 min and constant 80% B for 5 min. A third gradient method for the joint analysis of both extracts was created to study the partition of the extracted compounds between the two immiscible phases. This gradient was as follows: 4% B until min 10; change from 4% to 20% B in 15 min, from 20% to 60% B in 30 min, from 60% to 100% B in 40 min and constant 100% B for 10 min. The selected wavelengths for monitoring were 210, 270 and 320 nm.

2.7. LC–QTOF MS/MS analysis

The same extracts prepared for LC–DAD analysis and the same chromatographic gradients were used for LC–QTOF characterization. Deionized water with 0.1% v/v of formic acid (mobile phase A) and ACN with 0.1% formic acid v/v (mobile phase B) were used. Five μL of sample was injected from the autosampler thermostated at 5 ºC. Dual electrospray ionization (ESI) source and mass spectrometer parameters, operating in negative and positive ionization modes, were as follows: capillary, skimmer and fragmentor voltage were set at +/– 3.5 kV, 65 V and 175 V, respectively; N_2 nebulizer gas was flowed at 35 psi; N_2 drying gas flow rate and temperature were 10 L/min and 350 °C; N_2 sheath gas flow rate and temperature were 10 L/min and 380 ºC; and octopole radiofrequency voltage was set at 750 V. The extracts were analyzed in positive and negative ionization modes and at two values of collision energy, 15 and 25 eV, using the centroid mode, and the auto MS/MS mode (*m/z* range 100–3000); then, the data were collected at one spectrum per second in the extended dynamic range mode. The auto MS/MS mode was configured with 2 maximum precursors per cycle and an exclusion window of 1 min after two consecutive selections of the same precursor. To assure the desired mass accuracy of recorded ions, continuous internal calibration was performed during analyses with the use of signals at *m/z* 121.0509 (protonated purine) and *m/z* 922.0098 [protonated hexakis (1H, 1H, 3H-tetrafluoropropoxy)phosphazine or HP-921] in positive ionization mode; and *m/z* 112.9856 (trifluoroacetic acid anion) and

m/z 1033.9881 (HP-921) in negative ionization mode.

2.8. Data treatment

Mass Hunter Workstation software (version B 05.00 Qualitative Analysis, Agilent Technologies, Santa Clara, CA, USA) was used to extract potential molecular features (MFs) from all the data sets. The extraction algorithm considered all ions exceeding 400 counts with a single charge. The isotopic distribution for inclusion of MFs should be defined by two or more ions (with a peak spacing tolerance of 0.0025 *m/z* and 10.0 ppm in mass accuracy). Adducts formation in positive (+H, +Na, +K, $+NH_4$) and negative ionization (-H, +Cl) modes, as well as neutral loss by dehydration were included to identify MFs corresponding to the same potential metabolite. Data files were treated in different groups, according to the extract (polar or non-polar) and the ionization mode (positive or negative). A baseline correction was done to eliminate background noise, and each set of raw data was filtered with a minimum counts level of 5000 (polar positive), 1800 (polar negative), 3000 (non-polar positive) and 1500 (non-polar negative). In the next step, the data sets were aligned with a tolerance window of 4 min in t_R values and 10 ppm in *m/z* values. The data matrix was exported into the Mass Profiler Professional (MPP) software package (version 2.0, Agilent Technologies, Santa Clara, CA, USA) for statistical analysis.

The MFs providing the highest chromatographic peaks were tentatively identified by searching MS and MS/MS information on different databases such as METLIN (http://metlin.scripps.edu/) and PlantCyc (http://plantcyc.org/). The selected precursor ions for identification were +H, +Na and $+H\text{-}H_2O$ aducts for positive precursor ions and –H and +Cl aducts for negative precursor ions, establishing an error limit of 10 ppm for identification.

3. Results and discussion

3.1. Preliminary studies

Before starting the optimization study of USAEE as such, preliminary tests were carried out by combination of different aqueous mixtures of organic solvents with *n*-hexane to form emulsions. In this preliminary test, orange peel was extracted by using the protocol described by Pérez-Serradilla *et al.* [30], who optimized the extractant composition by using aqueous mixtures of methanol in *n*-hexane to extract polar and non-polar compounds from different types of vegetal raw materials. They found an extractant composition 9:36:55 (v/v/v) water–methanol–*n*-hexane as optimum for extraction after emulsion formation. In the research here presented, three different aqueous mixtures (*viz.* 20:80 (v/v) water–organic solvent with methanol, ethanol or ACN) were assayed to be combined with *n*-hexane (45:55 aqueous mixture–*n*-hexane) in order to find the optimum composition for isolation of polar and non-polar compounds from orange peel. The hydroalcoholic mixtures provided the most stable emulsions under ultrasound application. The resulting extracts were separated by centrifugation and subsequently analyzed by LC–DAD, as described in the experimental section. Supplementary Fig. 1 shows the chromatograms from the extracts obtained using the three extractant compositions. The water–methanol mixture provided the best extraction efficiency in terms of number and intensity of chromatographic peaks; therefore, it was selected as polar extractant. Concerning the *n*-hexane phase, this solvent forms stable emulsions and it was suited for isolation of non-polar compounds.

3.2. Optimization of the USAEE

Once the extractant composition was set, the optimization study was focused on the US-assisted process to determine the values of US parameters to obtain the highest extraction efficiency. As described in the Materials and methods section, a response surface methodology was used for this purpose. Independent studies were carried out for polar and non-polar extracts resulting in the optimum conditions described in Table

1. The optimum extraction time was common to both types of extracts (8 min). Differences were observed for the other two parameters, sonication amplitude and duty cycle, as the optimum sonication amplitude was 43 *versus* 31%; while the duty cycle was 0.9 *versus* 0.69 s/s for non-polar and polar extracts, respectively. The differences at statistical level can be visualized in the response surfaces illustrated in Fig. 1 (A and B). The next step was to create a desirability response surface with capability to define the optimum conditions to obtain both extracts with the highest efficiency, which yielded the conditions listed in Table 1, while Fig. 2 shows the response surface reported by this study. As can be seen in Fig. 2, the desirable amplitude was lowered to 32% of the nominal value, while the duty cycle adopted an intermediate value (0.75 s/s). It is worth emphasizing that sonication amplitudes lower than 15% did not produced emulsion, thus decreasing the efficiency of the extraction process. Concerning the extraction time, optimum efficiency was attained after extraction for 7.5 min. Chromatograms of the extracts obtained under optimum and desirability conditions were compared and no visual differences were observed between both, as can be seen in Fig. 3, where the chromatographic peaks used for optimization of the US-assisted process are within the oval spaces. For this reason, the optimum extraction time found with the desirability study (7.5 min) was adopted.

Table 1. Optimization of the USAEE of polar and non-polar compounds from orange peel by independent monitoring of the non-polar phase, the polar phase and a desirability study to maximize the isolation of the two extracts.

Variable	Lower value	Higher value	Desirability conditions	Optimum conditions	
				Non-polar phase	Polar phase
Amplitude (%)	20.0	50.0	32.0	43.0	31.0
Duty cycle (s/s)	0.50	0.90	0.75	0.90	0.69
Time (min)	2.0	8.0	7.5	8.0	8.0

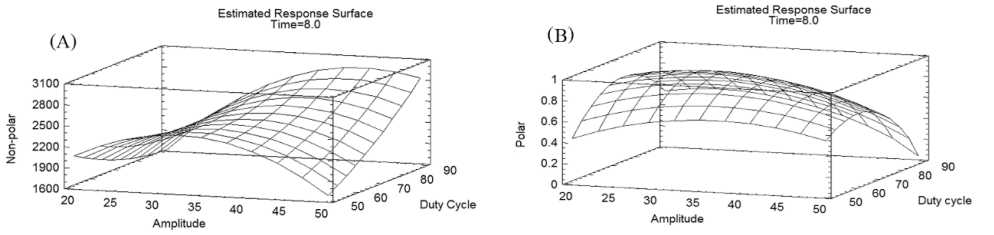

Figure 1. Response surfaces associated to the experimental design for USAEE of non-polar (A) and polar (B) compounds. Optimization was carried out by monitoring three representative chromatographic peaks obtained by LC–DAD analysis of the polar and non-polar extracts.

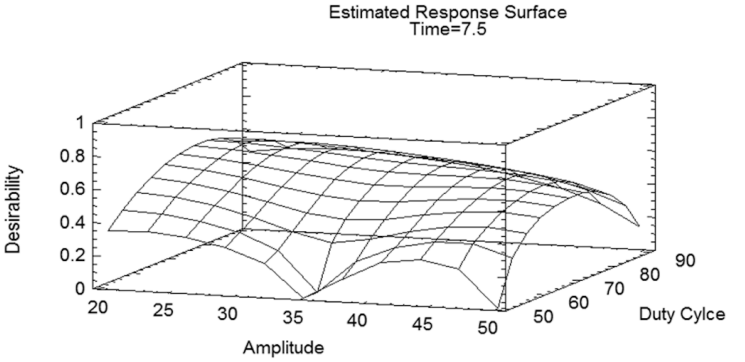

Figure 2. Response surface associated to the desirability conditions for USAEE. Optimization was carried out by monitoring three representative chromatographic peaks obtained by LC–DAD analysis of the polar and non-polar extracts.

An additional test to check the partition of the extracted compounds between the two phases was carried out by comparison of the LC–DAD chromatograms provided by the polar and non-polar USAE extracts analyzed by the same chromatographic method (Fig. 4). This test allowed comparison of the distribution of compounds detected in both types of extracts. The chromatogram provided by the polar extract was characterized by elution of most peaks before 40 min. The chromatogram from the non-polar extract provided some exclusive chromatographic signals in the elution window from 35-to-85 min, which were not detected in the analysis of the polar extract. Taking into account the reverse-phase gradient used in the chromatographic method, non-polar compounds with lipid character were isolated in the hexane phase. It is worth

mentioning that the peaks eluted in the time window 35–40 min were present in both extracts; therefore, only partition of them between both immiscible phases occurs. This orthogonal behavior justifies the potential use of USAEE for isolation of metabolites from orange peel with fractionation. As can be seen, the polar extract was also 1:30 diluted (Fig. 4B) in order to have a similar scale in terms of peak intensity to that provided by the non-polar extract, which was injected as such in the LC system.

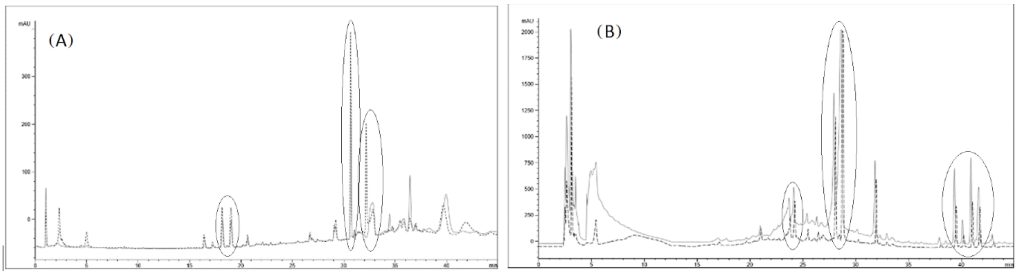

Figure 3. LC–DAD chromatograms from the non-polar (A) and polar (B) extracts obtained under optimum (continuous line) and desirability conditions (dotted line). Peaks within oval spaces have been used to monitor the optimization of the sample preparation step. The non-polar extract was not diluted and the polar extract was 1:2 diluted.

3.3. Comparison of the USAEE method with an extraction method without US-assistance

The USAEE under optimum conditions (7.5 min) was compared with a method using the same working conditions but without US application (that is, by simple maceration) and using different extractions times: 7 and 30 min, and 4 and 10 h. The resulting extracts were analyzed by LC–QTOF MS/MS in positive and negative ionization modes. The molecular features detected in each set of experiments were used to build a data set for statistical analysis based on PCA, which allowed overall comparison of extracts composition according to the ionization mode. Fig. 5 shows the PCA scores plots obtained from the polar and non-polar extracts obtained in the presence and absence of US, which allowed detecting the influence of US application on the extracts. In all cases, two-dimensional plots were used for this comparison by selecting the two principal components representing the maximum variability, which was always close or above 50% of the total variability. Comparison of the non-polar

extracts analyzed in negative ionization mode (Fig. 5A) revealed that PC1 allowed discriminating the extracts obtained at different extraction times. Therefore, the composition of US-assisted extracts was more similar to those of conventional maceration for 7 and 30 min and 4 h. Concerning PC2, this component showed a variability according to the processing time for conventional maceration, while US-assisted extracts were more similar to maceration for 10 h. The positive ionization mode reported a different scenario as compared to the negative mode for the non-polar extract (Fig. 5B). Thus, PC1 allowed detecting differences in the composition of US-assisted extracts as compared to those provided by conventional maceration. Component PC2 described a variability associated to the extraction time for conventional maceration and, in this context, US-assisted extracts were more similar to those obtained after extraction for 10 h. The different behavior found for positive and negative ionization modes should be attributed to the contribution of different families of compounds that are better detected in one of the ionization modes.

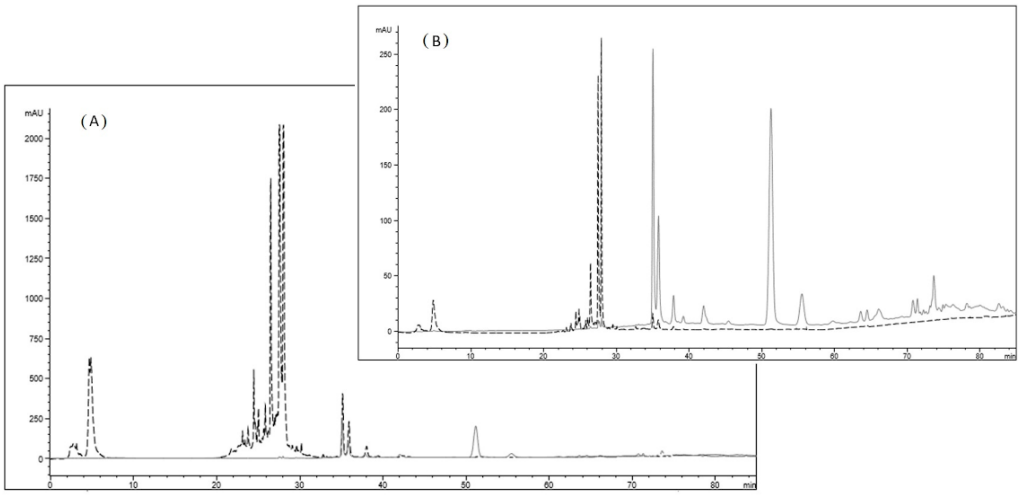

Figure 4. LC–DAD chromatogram provided by analysis of the polar (dotted line) and non-polar (continuous line) extracts using the same chromatographic method. The polar extract was 1:2 and 1:30 diluted in (A) and (B), respectively.

The polar extract in negative ionization mode enabled to deduce that the composition of the US-assisted extracts was similar to that of the conventional maceration for 4 h (Fig. 5C). The same result was observed in positive ionization mode by evaluating the variability ascribed to PC1 (57.3%). On the other hand, the variability associated to PC2 revealed a separation between US-assisted extracts and those obtained by maceration (Fig. 5D).

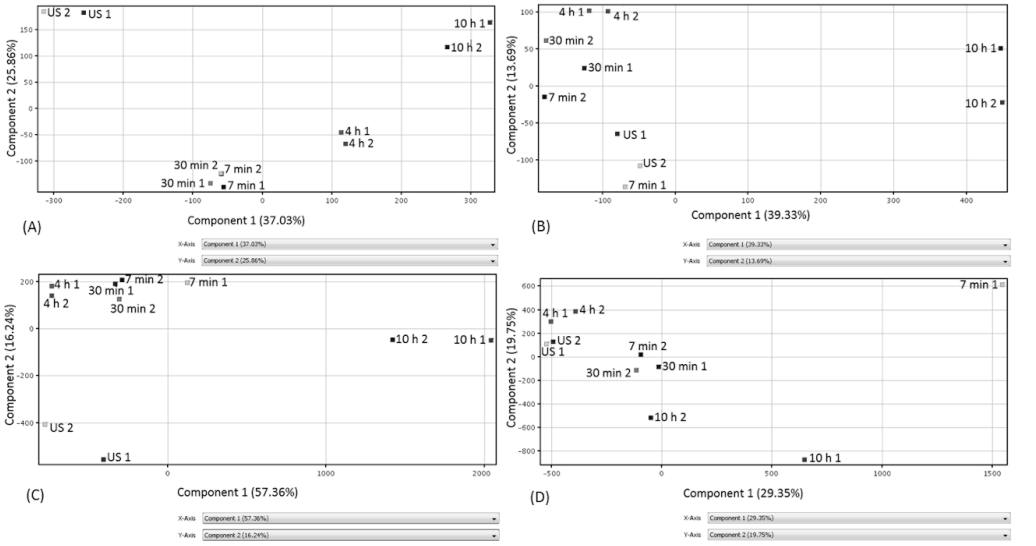

Figure 5. PCA scores plots describing the similarity between extracts obtained by USAEE and the conventional protocol applied for different extraction times (represented by number of min or h). The data set was built by LC–QTOF analysis of the non-polar in positive (A) and negative ionization modes (B) and polar extracts in positive (C) and negative ionization modes (D). Numbers 1 and 2 correspond to extractions of the same type of sample.

3.4. LC–QTOF MS/MS analysis

Polar and non-polar extracts obtained under the desirability conditions were analyzed by LC–QTOF MS/MS as described in the experimental section. Tentative identification of the most intense peaks was carried out by using MS/MS mode in high resolution for precursor and product ions (accuracy error < 6 ppm). Table 2 lists the tentative identified compounds classified by families as well as the main parameters

supporting the tentative identification. As can be seen, most metabolites identified in the polar extract were grouped in two families: coumarins and flavonoids, which are marked by their isomeric character. Thus, a group of coumarin isomers with the same *m/z* value as the precursor ion (261.1125 *m/z*) eluted within the retention time window 29–39.8 min. This group was also characterized by the presence of the same product ions (131.0491, 159.0432 and 189.0535 *m/z*), which are representative fragments obtained by MS/MS activation of the coumarin structure. Fig. 6 illustrates the extracted ion chromatogram for this precursor ion and also the MS/MS spectrum of one of the most intense peaks (eluting at 36.33 min) showing the most representative product ions. Apart from these isomeric coumarins, it should be emphasized the presence of other two coumarins, osthol and bergapten, which were properly identified attending to their MS/MS spectra, as illustrated in Supplementary Fig. 2. Coumarins were also identified in the non-polar extract, which is indicative of the partition between both extracts due to their high concentration as main pigment in orange peel.

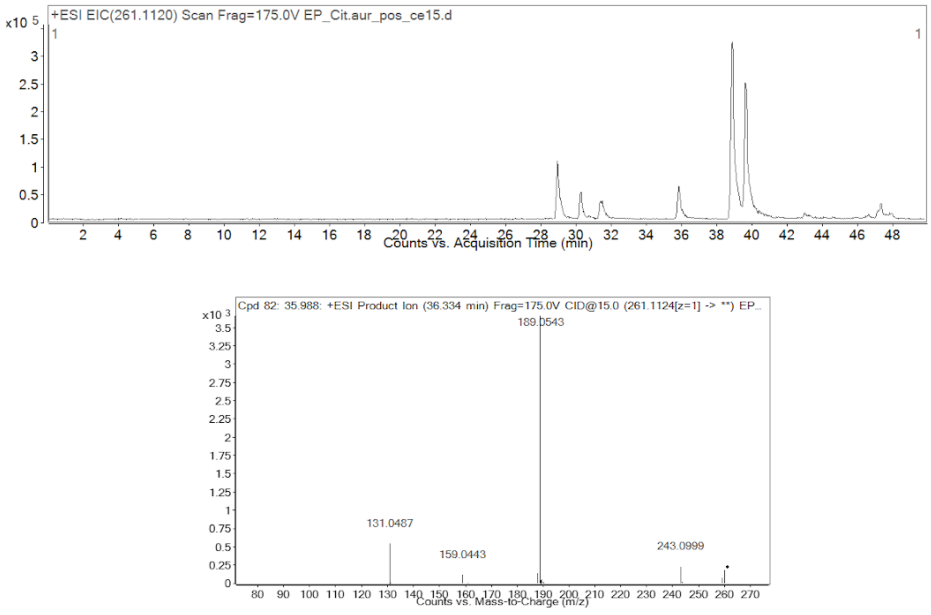

Figure 6. Extracted ion chromatogram (EIC) of the precursor ion with 261.1125 *m/z* identified as coumarin isomers and the MS/MS spectrum of the compound eluted at 36.3 min.

Concerning flavonoids, the main subfamilies of tentative identified compounds were polymethoxyflavones, flavonoid aglycones and glycosylated flavonoids. The last group encompassed flavonoid units condensed with one, two or three glycoside residues in different positions. Their isomeric character made unable their tentative identification. In this subgroup, one of the most intense peaks was identified as hesperidin; that is, a hesperetin molecule condensed with a diglucoside unit. Also, 4-methoxyplorizin, a derivate from plorizin, was detected in the polar extract. Glycosylated flavonoids were identified exclusively in the polar extract, which is quite logical since glycoside flavonoids are more polar than their respective aglycones. Hesperetin and naringenin, characteristic flavonoids in citrus fruits, were the two main aglycones tentatively identified in orange peel extracts. Concerning polymethoxyflavones, nobiletin, sinensetin and tangeretin, they were tentatively identified in both extracts, although their transfer into the polar phase was more favored. Other compounds, especially organic acids such as ascorbic acid or malic acid, were present at high concentration in polar extracts, and detected in the negative ionization mode.

In dealing with the non-polar extract, principal fatty acids were tentatively identified as exclusive compounds of this extract. Palmitic, stearic and lignoceric acids were the detected saturated fatty acids. Palmitic and stearic acids, common in plants, were present at low concentrations. Regarding to unsaturated fatty acids, oleic, linoleic and linolenic acids, the main unsaturated fatty acids present in vegetables and plants, were also identified in this extract.

Table 2. Compounds identified in polar and non-polar extracts of orange peel with their precursor and products ions.

Family	Compound	Ionization mode	m/z	RT (min)	Fragments	Error
Polar extract						
Coumarins	Coumarin	+	261.1121	31.5	189.0538, 131.0438	0
	Coumarin	+	261.1133	39.0	189.0548, 159.0432, 131.0494, 103.0542	4
	Coumarin	+	261.1131	39.8	189.0545, 159.0447, 131.0490, 103.0542	4
	Coumarin	+	261.1125	29.0	243.1000, 189.0535, 131.0479	0
	Coumarin	+	261.1123	30.3	189.0533, 159.0424, 131.0491	0
	Coumarin	+	261.1120	35.9	243.0994, 189.0539, 131.0479	0
	Osthol	+	245.1175	45.8	189.0546, 159.0434, 131.0492	1
	Bergapten	+	217.0494	39.6	202.0237, 174.0338, 161.0603, 131.0481	0
Polymethoxyflavones	Nobiletin	+	403.1394	41.3	373.0966, 388.1187	1
	Tangeretin	+	373.1287	43.3	358.1050, 343.0812, 325.0771	1
	Sinensetin	+	373.1280	39.9	357.0932, 343.0786, 329.1000	1
	Polymethoxyflavone	+	373.1291	38.5	343.0791, 329.0098	1
Flavonoid aglycones	Naringenin	+	273.0760	29.1	153.0179, 147.0430	0

Cont. Table 1

	Hesperetin	+	303.0868	29.8	285.0769, 262.0823, 177.0552, 153.0187	1
	4-Methoxyphlorizin	-	449.1465	28.8	402.7468, 227.0712, 151.0014	4
	Flavonoid + 1 glucoside	+	449.1446	29.8	413.1230, 328.0853, 263.0537, 165.0174	0
	Neohesperidin	+	611.1977	29.8	413.1231, 345.0970, 303.0860, 263.0539	1
		-	609.1852	27.6	489.1433, 381.1389, 301.0708	4
	Flavonoid + 2 glucosides	+	595.1654	27.7	541.1332, 457.1118, 409.0926, 325.0678	0
	Flavonoid + 2 glucosides	+	579.1711	29.1	463.1570, 383.1134, 315.0789, 271.0610	0
	Flavonoid + 2 glucosides	+	581.1874	29.1	395.0748, 329.0622, 273.0780, 147.0647	1
Glycosylated flavonoids	Flavonoid + 2 glucosides	-	579.1745	24.2	459.1164, 373.0927, 271.0609, 151.0029	6
	Flavonoid + 2 glucosides	-	595.1692	26.2	459.1143, 287.0558, 151.0032	3
	Flavonoid + 2 glucosides	-	593.1528	2.5	473.1090, 353.0624, 270.0522, 173.0052	2
	Flavonoid + 2 glucosides	-	577.1572	28.0	463.1142, 362.6551, 271.0617	1
	Flavonoid + 3 glucosides	+	755.2394	31.0	369.0945, 345.0947, 303.0880, 145.0494	0
	Flavonoid + 3 glucosides	-	753.2278	30.0	691.2221, 651.1928, 609.1824	4
	Flavonoid + 3 glucosides	-	723.2168	29.2	621.1832, 579.1727, 271.0598	3
	Flavonoid + 1 glucoside + 2 coumarin	-	723.1765	27.5	610.1910, 222.9127, 112.9855	6

Cont. Table 1

Others	Isocitrate	-	191.0203	2.3	173.0076, 147.0285, 129.0189, 111.0084	2
	Ascorbic acid	-	175.0251	2.3	115.0040, 126.1021, 152.1718	1
	Malic acid	-	133.0144	2.1	129.9507, 115.0035, 103.4415	1
	2-Furoic acid	-	111.0086	2.9	-	1
	Quinic acid	-	191.0566	2.3	173.0451, 155.0341, 127.0412, 109.0284	2
	Disacharide	-	341.1095	2.7	297.6323, 237.6446, 179.0565, 131.0458	1
	Prolinebetaine	+	144.1023	3.0	116.106	2
Non-polar extract						
Coumarins	Coumarin	+	261.1123	25.7	219.1732, 189.0537, 131.0487	0
	Coumarin	+	261.1121	8.9	189.0538, 131.0438	0
	Coumarin	+	261.1133	15.9	189.0548, 159.0432, 131.0494, 103.0542	4
	Coumarin	+	261.1131	16.8	189.0545, 159.0447, 131.0490, 103.0542	4
	Osthol	+	245.1175	23.8	189.0546, 161.0590, 131.0492	1
	Bergapten	+	217.0494	16.9	202.0237, 187.0430, 161.0603, 131.0481	0
Polymethoxyflavones	Nobiletin	+	403.1394	18.5	373.0966, 388.1187	1
	Tangeretin	+	373.1287	20.9	358.1050, 343.0812, 297.0760	1
	Sinensetin	+	373.1280	16.7	357.0933, 343.0782, 329.1006	1

Cont. Table 1

Fatty acids						
Palmitic acid	-	255.2327	31.1	-	4
Stearic acid	-	283.2640	38.7	-	7
Oleic acid	-	281.2477	38.0	-	0
Linoleic acid	-	279.2338	32.9	-	3
Linolenic acid	-	277.2171	30.9	-	0
Lignoceric acid	-	367.3585	42.1	-	2

4. Conclusions

USAEE provided combined, simultaneous extraction of polar and non-polar compounds present in the sample in a single step. Moreover, the time required to obtain the extracts was much shorter than that needed by conventional extraction methods, thus making USAEE a rapid sample preparation step prior to metabolic profile analysis. The entities analyzed in each extract showed that orange peel is rich in high-priced metabolites, with a higher number of compounds in polar extracts than in non-polar extracts. Tentative identification of the metabolites showed numerous compounds with a high-added value, such as flavonoids (hesperidin, neohespridin, naringin, tangeretin), fatty acids (linolenic acid, linoleic acid, oleic acid) and organic acids (citric acid, malic acid, quinic acid) present in orange peel. These results open a door toward completion of the metabolite profile of orange peel, identification of minor compounds in it, studies of the degradation processes and the degraded compounds generated during the drying step. This could lead to identification of compounds with high-added value and development of effective extraction methods to obtain them.

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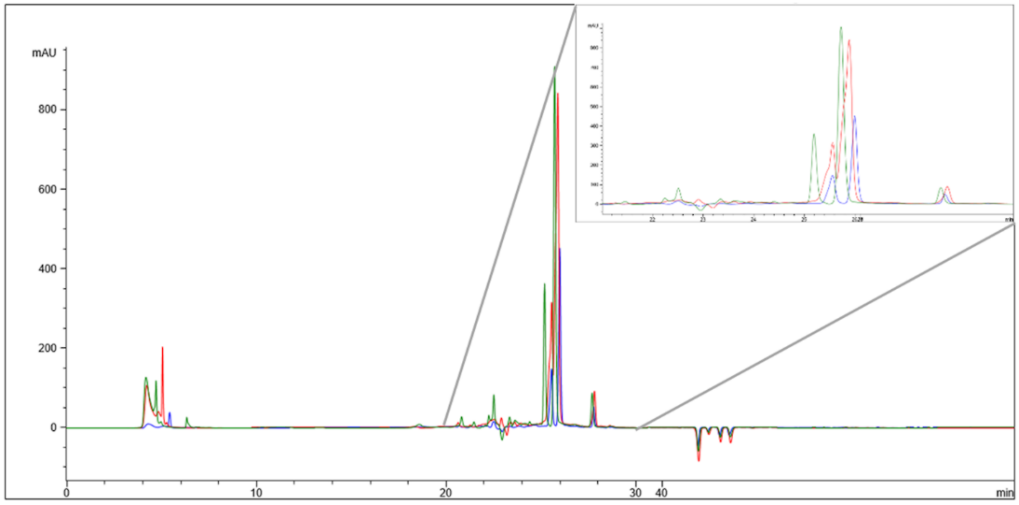
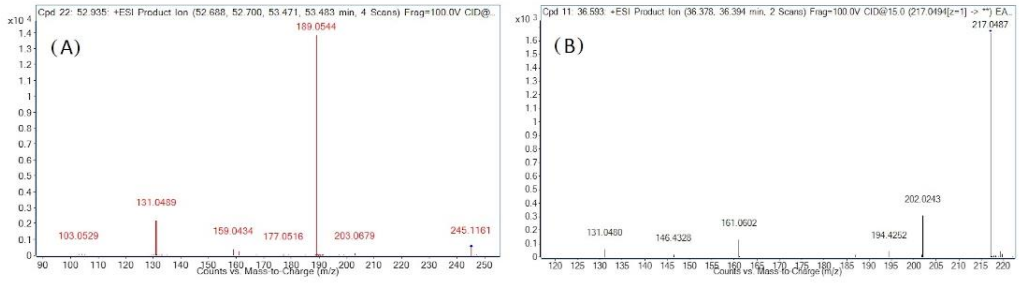
Supplementary Information



Supplementary Figure 1. LC–DAD chromatograms provided by analysis of polar extracts obtained by the aqueous mixtures 80:20 (v/v) organic solvent–water. Ethanol (red), methanol (green) and acetonitrile (blue). The x-axis in the central part of the chromatograms has been expanded for better distinction of the differences in the peaks within this interval.



Supplementary Figure 2. MS/MS spectra of osthol (A) and bergapten (B).

CAPÍTULO III

Development and application of a quantitative method for determination of flavonoids in orange peel: Influence of sample pretreatment on composition

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Development and application of a quantitative method for determination of flavonoids in orange peel: Influence of sample pretreatment on composition

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Development and application of a quantitative method for determination of flavonoids in orange peel: Influence of sample pretreatment on composition

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ABSTRACT

Peel, a part of the citrus rich in compounds with high-added value, constitutes the bulk of the waste generated in citrus juice industries. Flavonoids are a class of these high-added value compounds characterized by their bioactivity. In this research, a method for analysis of flavonoids, based on LC–MS/MS by using a triple quadrupole detector, has been developed and applied to the quantitative analysis of 16 flavonoids in extracts obtained by maceration of citrus peel. The parameters involved in the ionization and fragmentation of the target analytes were optimized to develop a selected reaction monitoring (SRM) method, which reported detection and quantitation limits ranging from 0.005 to 5 ng/mL and from 0.01 to 10 ng/mL, respectively. The raw materials for flavonoids extraction were fresh, oven-dried and lyophilized peel of 8 different orange varieties, and the proposed quantitation method was applied to the analysis of the extracts. Evaluation of the two methods of water removal showed that lyophilization preserves the concentration of the flavonoids, while oven-dried peel presented a decrease of glycosylated flavonoids and an increase of aglycone forms.

Keywords Citrus peel; flavonoids; quantitation; triple quadrupole; lyophilization; oven-dry

1. Introduction

A significant part of the world harvest of citrus is destined to production of citrus juices, generating tons of residues formed by peel (constituted by flavedo and albedo, the non-edible parts of the fruit), and seeds [1]. In traditional agriculture and production, these residues have low or nil value and even they can constitute an environmental problem owing to their accumulation close to industrial zones. The generated residues have traditionally been transformed into pelletized bran for animal feeds [2], despite this material is rich in compounds that could be isolated for different applications. In dealing with the most representative types of cultivars in the Mediterranean area (typically olive trees and vineyards), large amounts of residues are generated (*e.g.*, lees, grape skins, seeds and stalks in the case of wine production, and olive pomace and leaves in olive oil production), which are in the way for integral exploitation to obtain secondary high-valued products and facilitate the use of the residues, while achieving landfills decrease [3].

A class of phenolic compounds with potential nutraceutical benefits because of their described antioxidant activity [4] is formed by flavonoids, the proportion of which in citrus peel is high enough for potential interest of this material to obtain these compounds. Previous studies have attributed to flavonoids anti-inflammatory and antimicrobial activity [4,5] that could explain their potential prevention role against atherosclerosis and cancer [6–9]. There are many sub-classes of flavonoids in citrus fruits, among which flavanones are a major one [11], in turn classified into glycoside and aglycone flavanones depending on they have or not a glycoside group in their structure. The most abundant glycoside flavanones in citrus peel are naringin, hesperidin and neohesperidin that presumably exhibit health benefits such as suppression of carcinogenesis and decay in proliferation of cancer cells [11,12]. On the other hand, some studies have argued that aglycone flavonoids exhibit higher antioxidant capacity and radical

scavenger efficiency than their respective glycosides [14], but their concentration in these fruits is lower than that of glycoside flavonoids. Polymethoxyflavones constitute a peculiar sub-class within flavonoids because their structure and presence, almost exclusive of citrus [15]. Several studies attribute a broad spectrum of biological activity to these flavonoids, including anti-inflammatory [16], anti-carcinogenic [17] and anti-atherogenic properties [18].

Different methods have been reported for analysis of flavonoids in foods. Most of the researchers working in this area have determined total phenols using the Folin-Ciocalteu reagent by which the total content of phenolic acids is jointly determined. This reagent also interacts with other non-phenolic compounds such as ascorbic acid, leading to an overestimation of the total phenol content [19]. Liquid chromatography (LC) is widely used for flavonoids separation prior to detection, being capillary electrophoresis (CE) used in a lower proportion. Most times, LC has been coupled to diode array detection (DAD) for the individual determination of flavonoids [20], thus requiring standards for identification of target compounds, and providing low sensitivity as compared with mass spectrometry (MS) detectors. The latter are at present the preferred option as they joint high resolution and sensitivity, allowing identification and quantitation of flavonoids. As examples, Liu *et al.* and Ćirić *et al.* have developed methods based on the coupling of a triple quadrupole (QqQ) mass spectrometer to a liquid chromatograph for quantitative analysis of five and six of the main flavonoids, respectively, in orange peel [20,21]. A more ambitious research was developed by Delpino-Rius *et al.*, who proposed a method for quantitation of fourteen flavonoids in processed fibers from the citrus juice industry by LC–DAD/Fluorescence detection with confirmatory analysis by LC–QqQ MS/MS [23]. Similarly, LC–DAD–QqQ MS/MS coupling was critical in the study carried out by Abad-García *et al.*, who identified and relatively quantified 58 phenolic compounds in juices from orange, tangerine, lemon and grapefruit, including 54 flavonoids [24].

Despite several studies have dealt with quantitation of flavonoids in citrus peel, none of them has involved more than three of four citrus varieties or has evaluated the effect of the water-removal step on the concentration of flavonoids. Thus, the main objectives of the present research were to develop an LC–MS/MS based-method for quantitation of sixteen flavonoids for application to the analysis of extracts from peels of eight orange varieties. Lyophilization and oven-dry were compared to estimate the influence of both sample pre-treatment strategies on the concentration of target flavonoids in each orange variety.

2. Materials and methods

2.1. Samples

The orange varieties under study were Salustiana, Navelina, Navel Lane Late and Sanguinelli oranges, which belong to the specie *Citrus sinensis*; Tangerin (*Citrus reticulata*); Clemenules clementine and Hernandina clementine (subspecie *Citrus clementine* of *Citrus reticulata*); and bitter orange (*Citrus aurantium*). All varieties were from Spanish citrus crops and were purchased in a local supermarket, excluding bitter orange, which was collected from ornamental trees in the campus of the University of Córdoba.

2.2. Extraction method

The peel of two-to-four oranges (depending on the size of the given variety) was shredded and the resulting grated was divided into three fractions: one of them remained fresh and was immediately extracted, the second was lyophilized and the third part was oven-dried at 35 ^{o}C for 3 days. 0.5 g of each fraction and each variety was extracted with 15 mL of methanol for 7 min with the assistance of a magnetic stirrer working at 400 rpm. The obtained extracts were filtered by a 0.45 μm filter and stored at –20 ^{o}C until analysis. The extracts were 1:10 and 1:100 (v/v) diluted in methanol for analysis.

2.3. Standards and reagents

Supplementary Table 1 lists the flavonoids analyzed in this research classified by their structure and the flavone unit. Kaempferol, neohesperidin, luteolin, luteolin-7-O-glucoside, homoorientin, tangeretin, diosmetin and formononetin were from Extrasynthese (Genay, France); quercetin, and hesperidin were from Fisher (Madrid, Spain); and hesperetin, kaempferol-7-O-glucoside, apigenin-7-O-neohesperidoside, apigenin, naringenin, naringin and oleuropein were from Sigma–Aldrich (St. Louis, USA). All reagents were analytical grade or higher. Deionized water (18 MΩ·cm) was obtained by a Mili-Q water purification system from a Millipore (Bedfore, MA, USA) Milli-Q plus system; LC grade methanol was from Panreac (Barcelona, Spain) and LC grade acetonitrile (ACN) and formic acid were from Scharlab (Barcelona, Spain).

2.4. Apparatus and instruments

An Agilent 1200 Series LC system from Agilent Technologies (Palo Alto, USA) with a 6460 Triple Quad LC–MS detector equipped with Jet Stream Technology electrospray ion source, also from Agilent, was used to carry out the analyses. A Mediterranea Sea C18 analytical column (3 µm, 15 × 0.46 cm) from Teknokroma (Barcelona, Spain) was used together with deionized water containing 0.1% of formic acid (mobile phase A), and ACN also with 0.1% of formic acid (mobile phase B). The gradient for analysis of the extract was as follows: 25% B until 2 min, change from 25% to 30% B in 2 min, from 30% to 32% B in 4 min, from 32% to 55% B in 25 min and constant 55% B for 2 min more. A post-run of 7 min was programmed to equilibrate the column between analyses. The flow rate was constant at 0.8 mL/min and the injected volume was 10 µL.

High-purity nitrogen (99.999%) as collision gas was used in the MS triple quadrupole detector. Determination of the compounds was performed by ESI-MS/MS in selected reaction monitoring (SRM) mode. The sheath gas flow and temperature were established at 12 L/min and 375 ºC, respectively, and the gas

temperature was set at 325 ºC. The pressure of the nebulizer was 45 psi, the capillary voltage was established at 2000 V for both ionization modes and the nozzle voltage at 500 V. The dwell time was set within the range 75–200 ms for each SRM transition, depending on the chromatogram segment.

2.5. Quantitation of flavonoids

The calibration curves were run by plotting the ratio between the peak area of each flavonoid and that of the internal standard *versus* its nominal concentration in the multistandard solutions. For this purpose, 12 calibration levels were prepared in methanol with the target analytes at suited concentrations to define the calibration ranges and evaluate the sensitivity of the method by estimation of the limits of detection and quantitation (LODs and LOQs, respectively). Each calibration level as well as the samples were spiked with 10 µL of a solution of oleuropein at a concentration of 10 µg/mL for its use as internal standard and their analyses were performed in triplicate.

3. Results and discussion

3.1. Optimization of the SRM detection mode

The target flavonoids were selected as they are widely described in the literature in dealing with orange peel. The main parameters involved in MS ionization and fragmentation were optimized to achieve the highest sensitivity in detection of the selected compounds. The ionization operating conditions were studied by direct injection of standard solutions of each individual analyte at 5 µg/mL using both positive and negative ionization modes. Quantitation of each flavonoid was based on the SRM mode. MS/MS parameters were optimized for efficient isolation of the precursor ions and their sensitive and selective fragmentations. Voltage of the first quadrupole for an efficient filtration of the precursor ion, collision energy and selected product ions for the quantitation

transition are listed in Table 1. The negative ionization mode provided better sensitivity for the determination of all flavonoids under study, excluding tangeretin, kaempferol and kaempferol-7-O-glucoside, the determination of which provided better sensitivity in the positive ionization mode. Negative ionization led to generation of $[M\text{-}Rha]^-$ precursor for apigenin-7-O-neohesperidoside and $[M–H]^-$ precursor for the rest of flavonoids; on the other hand, the positive ionization of tangeretin, kaempferol and kaempferol-7-O-glucoside generated the $[M+H]^+$ precursors. The SRM transition of the glycosylated flavonoids was produced by loss of the glycoside moiety, excluding homoorientin transition, the glycoside unit of which was intramolecularly rearranged to form the product ion shown in Fig. 1. The aglycone flavonoids quercetin, apigenin, kaempferol, luteolin and naringenin followed the same fragmentation pattern resulting in the fragment 150.9 *m/z* in negative and 152.9 *m/z* in positive ionization modes, while hesperetin, diosmetin and formononetin presented different fragmentation patterns (see Fig. 2 and Supplementary Fig. 1).

After selection of parameters for each SRM transition, the variables of the ESI source were optimized as show the results listed in Table 2. Particularly, the ionization of the target analytes demanded gas temperature (N_2 for nebulization of the eluate from the chromatograph) and sheath gas temperature (N_2 for focalization of the nebulized flow) above 300 ^oC, characteristic of non-thermolabile compounds. The capillary voltage was set at 2 kV and the nozzle voltage was set at 0.5 kV, values below the intermediate voltages, thus indicating the facility of ionization of the target compounds.

Finally, the resulting SRM chromatograms of the multistandard and two different samples are shown in Supplementary Fig. 2, where peaks corresponding to each flavonoid are numbered.

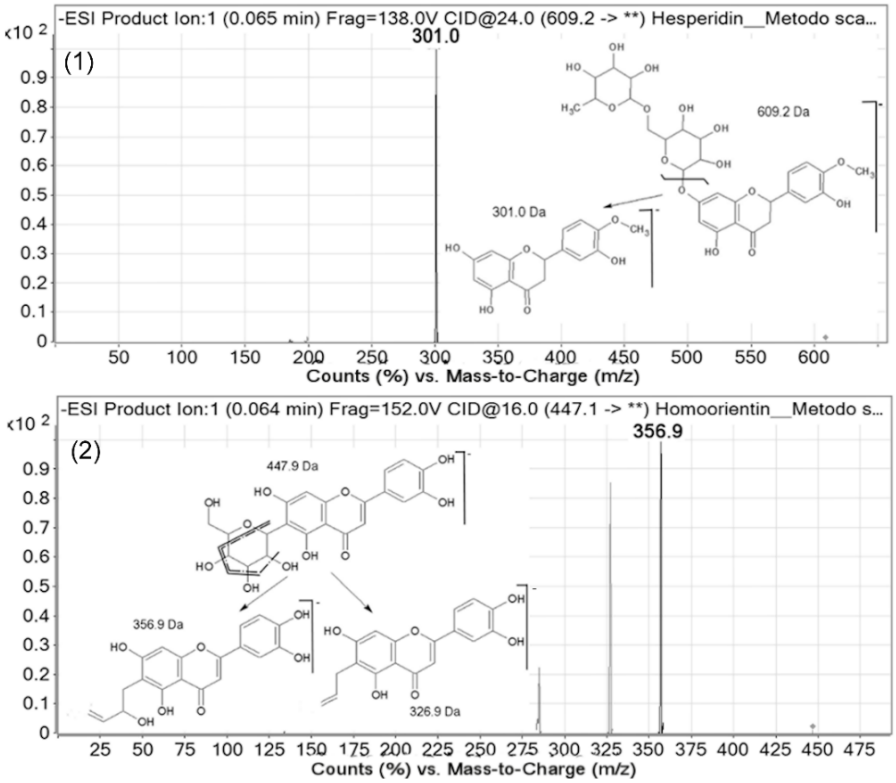

Figure 1. MS/MS spectra and fragmentation patterns of hesperidin (1) and homoorientin (2).

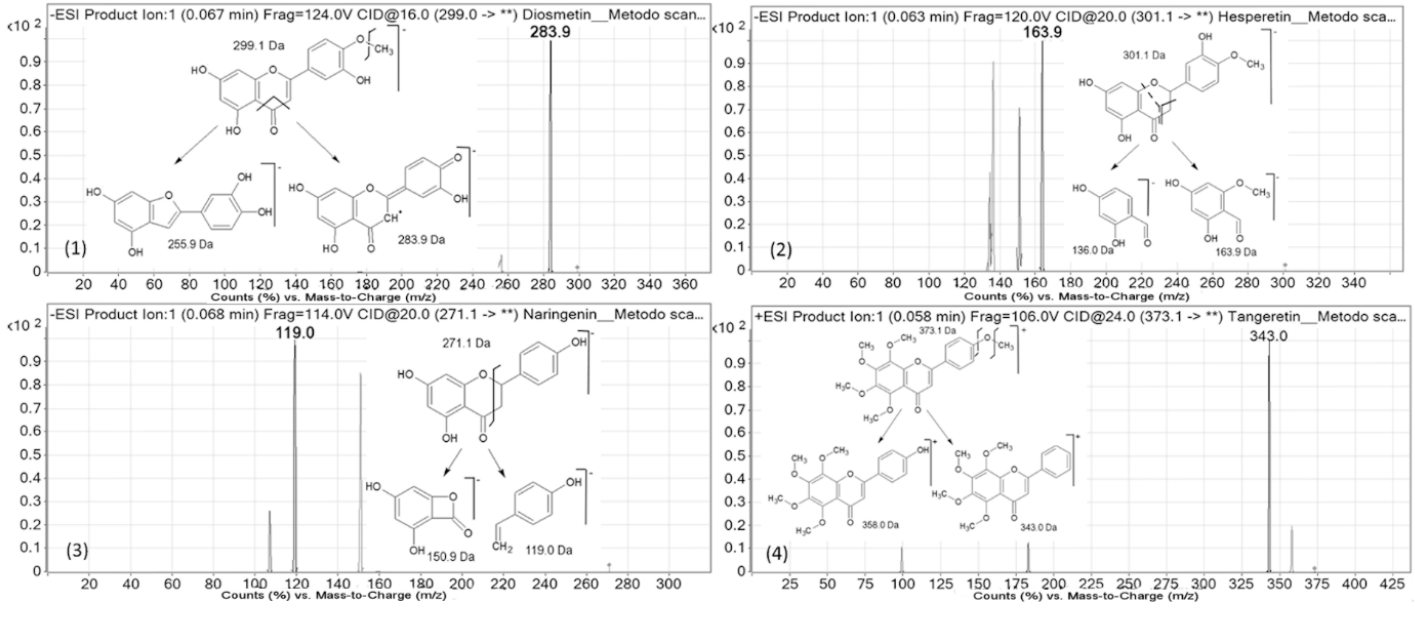

Figure 2. MS/MS spectra and fragmentation patterns of diosmetin (1), hesperetin (2), naringenin (3) and tangeretin (4).

Table 1. Parameters of the LC–MS/MS method for qualitative and quantitative determination of the target flavonoids and their retention times.

Compound	Ionization mode	Precursor ion (*m*/*z*)	Product ions (*m*/*z*)	Quantitation transition	Quadrupole voltaje (V)	Collision energy (eV)	Retention time (min)
Homoorientin	–	447.9	356.9, 326.9, 284.8, 132.9	447.9 → 356.9	152	16	3.5
Luteolin-7-O-glucoside	–	447.1	284.9, 217.1, 150.9, 132.9	447.1 → 284.9	178	24	5.4
Kaempferol-7-O-glucoside	+	286.9	286.9, 152.9, 92.9	449.1 → 286.9	176	16	6.5
Naringin	–	579.2	459.0, 270.9, 150.9, 119.0	579.2 → 150.9	198	48	6.6
Hesperidin	–	609.2	301.0, 198.3, 185.2	609.2 → 301.0	138	24	6.9
Neohesperidin	–	609.2	335.5, 301.0, 286.0, 271.1	609.2 → 286.0	180	44	7.7
Apigenin-7-O-neohesperidoside	–	577.5	267.9, 238.9, 210.9, 150.9	577.5 → 267.9	164	36	7.9
Luteolin	–	285.0	207.4, 206.1, 151.0, 132.9	285.0 → 132.9	134	32	15.8
Quercetin	–	301.0	183.2, 150.9, 120.9	301.0 → 150.9	118	16	16.1
Naringenin	–	271.1	160.0, 150.9, 119.0, 107.0	271.1 → 119.0	114	20	20.4
Apigenin	–	269.0	224.9, 151.0, 148.9, 117.0	269.0 → 117.0	134	32	21.1
Hesperetin	–	301.1	163.9, 150.9, 136.0, 134.0	301.1 → 136.0	120	28	21.6
Kaempferol	+	287.2	152.9, 92.9, 77.0	287.2 → 152.9	136	32	22.1
Diosmetin	–	299.1	283.9, 255.9, 176.0	299.1 → 283.9	124	16	22.1
Formononetin	–	267.1	251.9, 222.9, 194.9, 131.9	267.1 → 251.9	124	16	25.4
Tangeretin	+	373.1	358.0, 343.0, 182.9, 98.9	373.1 → 343.0	106	24	34.7

Table 2. Optimized ion source parameters for the proposed LC–MS/MS method.

Variable	Tested range	Optimal value
Gas temperature (°C)	0–350	350
Sheath gas temperature (°C)	0–400	375
Capillary voltage (V)	0–6000	2000
Nozzle voltage (V)	0–2000	500

3.2. Linearity of calibration curves and lower limits of detection and quantitation

A linear regression model was applied to generate two calibration curves within two different concentration ranges for each metabolite: from LOQ to 0.1 µg/mL and from 0.1 to 5.0 µg/mL. The regression coefficients (R^2) of the calibration models were higher than 0.99 in all instances, as Table 3 shows, indicative of an excellent fitting. The sensitivity of the method was evaluated by estimation of the LOD and LOQ for each analyte by injecting dilution series of a multistandard solution to obtain the concentration which provided signals three and ten times the background noise, respectively (Table 3). The LOQs for these flavonoids were within the range 0.01–10.00 ng/mL, while the LODs were within 0.005–5 ng/mL. It is noteworthy that tangeretin presented the lowest LOD and LOQ as compared to the rest of flavonoids since the product ion 343.0 *m/z* was generated by the loss of a methoxide group. The presence of five methoxide groups in the structure of tangeretin led to the generation of an intense fragment representative of this quantitation transition. On the other hand, differences based on the subclass were not appreciated in LODs and LOQs between glycoside and aglycone flavonoids. The sensitivity provided by the methods reported in the literature shows that Ćirić *et al*., who quantified five flavonoids in orange peel by LC–MS/MS triple quad, obtained LOQs and LODs within the range 121–168 ng/mL and 39–76 ng/mL, respectively; while Liu *et al*. reached LOQs and LODs

within the range 0.20–31.94 ng/mL and 0.05–9.58 ng/mL, respectively, also using LC–MS/MS triple quad for quantitation of six flavonoids. Comparing these values with those achieved by the method here proposed, the highest sensitivity for quantitation of the target flavonoids corresponds to the latter. Concerning the methods reported by Delpino-Rius *et al.* and Abad-García *et al.*, despite they analyzed a greater number of compounds than the method proposed here, they did not offer any information about LODs and LOQs since they were targeted at qualitative profiling and semiquantitative analysis.

3.3. Kinetics of the extraction procedure

Four extraction experiments from lyophilized Navel Lane Late orange peel were carried out at different extraction times to evaluate the influence of the processing time on the extraction of flavonoids. The assayed times were 7, 30, 240 min (4 h), and 600 min (10 h). Tangeretin, hesperetin and neohesperidin were selected as model compounds of the different families of flavonoids. Fig. 3 shows the extraction efficiency, expressed as normalized peak area, of these compounds *versus* the extraction time. As can be seen, a decrease of the extraction efficiency along the processing time can be observed. Seven and 30 min experiments resulted in a similar extraction yield, which means that flavonoids are rapidly extracted. Even extraction times longer than 4 h yielded extracts with a decreased relative concentration of the monitored flavonoids, probably owing to degradation. Therefore, 7 min was selected as suited extraction time.

Table 3. Analytical features of the method for determination of the target flavonoids.

Compound	Calibration 1 (LOQ–0.1 µg/mL)		Calibration 2 (0.1–5 µg/mL)		Limit of detection (ng/mL)	Limit of quantitation (ng/mL)
	Calibration equation	Regression coefficient	Calibration equation	Regression coefficient		
Tangeretin	y = (334.18 ± 5.61)x + (0.3656 ± 0.260)	0.9991	y = (212.72 ± 8.71)x + (27.833 ± 19.59)	0.9933	0.005	0.01
Kaempferol	y= (1.3853 ± 0.043)x – (0.0012 ± 0.002)	0.9982	y = (2.0866 0.059)± x – (0.0512 ± 0.133)	0.9968	5	10
Formononetin	y= (72.197 ± 0.812)x + (0.0209 ±0.037)	0.9997	y = (33.982 ± 1.823)x + (7.2612 ± 4.416)	0.9914	0.1	0.25
Quercetin	y= (0.2994 ± 0.148)x – (0.0006 ± 0.008)	0.9979	y = (5.7347 ± 0.217x – (1.538 ± 0.487)	0.9943	1	5
Naringenin	y = (10.175 ± 0.125)x + (0.0003 ± 0.005)	0.9996	y = (9.8105 ± 0.319)x + (0.3649 ± 0.717)	0.9958	0.5	1
Luteolin-7-O-glucoside	y = (9.1587 ± 0.366)x + (0.324 ± 0.017)	0.9956	y = (8.5082 ± 0.320)x + (0.9178 ± 0.721)	0.9944	0.5	1
Luteolin	y = (13.773 ± 0.176)x + (0.0044 ± 0.008)	0.9996	y = (10.662 ± 0.522)x + (1.5642 ± 1.173)	0.9952	1	5
Hesperetin	y = (1.5322 ± 0.031)x + (0.0011 ± 0.001)	0.9992	y = (1.4868 ± 0.051)x + (0.0696 ± 0.115)	0.9905	5	10
Neohesperidin	y= (0.3768 ± 0.006)x + (0.0007 ± 0.0003)	0.9995	y = (0.3985 ± 0.012)x + (0.0018 ± 0.029)	0.9970	5	10
Kaempferol-7-O-glucoside	y= (0.7469 ± 0.054)x + (0.0004 ± 0.0001)	0.9998	y= (0.9583 ± 0.024)x – (0.0191± 0.054)	0.9975	0.5	1
Homoorientin	y = (7.1647 ± 0.195)x + (0.0148 ± 0.010)	0.9979	y = (5.3513 ± 0.258)x + (0.7876 ± 0.579)	0.9908	0.5	1
Apigenin-7-O-glucoside	y = (30.326 ± 0.521)x + (0.0098 ± 0.013)	0.9991	y = (15.935 ± 0.690)x + (1.8314 ± 1.551)	0.9926	0.25	0.5
Naringin	y = (2.4453 ± 0.024)x + (0.0062 ± 0.001)	0.9998	y = (2.5872 ± 0.077)x + (0.0518 ± 0.172)	0.9965	0.5	1
Hesperidin	y= (2.4775 ± 0.052)x + (0.0052 ± 0.002)	0.9991	y = (3.2697 ± 0.066)x – (0.1657 ± 0.147)	0.9984	1	5
Apigenin	y = (27.4 ± 0.257)x + (0.0038 ± 0.012)	0.9998	y = (16.223 ± 0.657)x + (2.0809 ± 1.593)	0.9951	0.25	0.5
Diosmetin	y = (16.67 ± 0.207)x + (0.0079 ± 0.009)	0.9995	y = (9.0471 ± 0.411)x + (1.4369 ± 0.996)	0.9938	0.5	1

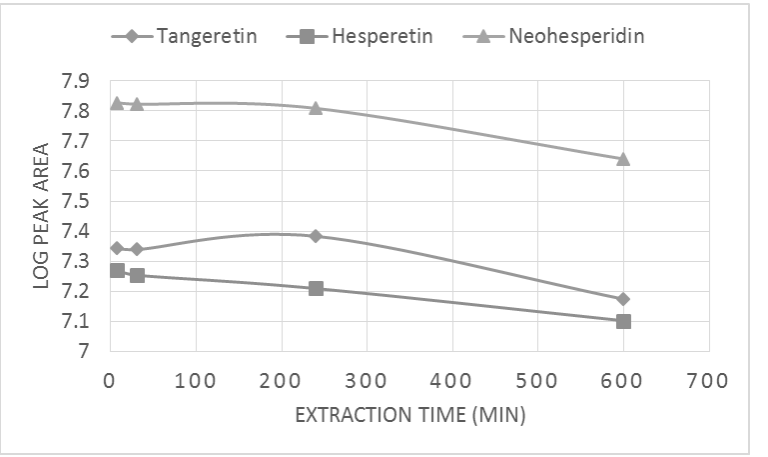

Figure 3. Extraction kinetics of tangeretin, hesperetin and neohesperidin expressed as normalized peak area by logarithmic transformation.

3.4. Determination of the target flavonoids in orange peels

The proposed LC–MS/MS method was applied to the analysis of the target flavonoids in the peel of different selected varieties of fresh, lyophilized and oven-dried oranges. The comparison of these experiments allowed evaluating the impact of the pretreatment strategy on the quantitative analysis of flavonoids in orange peel. Supplementary Table 2 lists the concentration of flavonoids found in the analyzed samples, expressed as ng/g of peel. Generally, glycoside flavonoids are present at higher concentration than the respective aglycone (see Fig. 4). This behavior can be explained by the fact that flavonoids are mainly stored in an aqueous medium inside the cells, thus the glycoside form is favored due to its higher polarity. It is worth mentioning that kaempferol and formononetin were not detected in the selected orange varieties. The concentration of target flavonoids present in fresh, lyophilized and oven-dried peels is represented by bar diagrams in Fig. 4 for each variety, where the flavonoids were grouped according to their concentration levels. As can be seen, neohesperidin, hesperidin and naringin, the three major flavonoids in the samples, were highly concentrated in lyophilized samples due to the removal of water content. Hesperidin was the major flavonoid

in all varieties, excluding bitter orange, where naringin and neohesperidin were the two major flavonoids, with concentrations above 10 µg/g of fresh peel. The increase of these compounds to the detriment of hesperidin in bitter orange can be explained by the flavonoid biosynthesis pathway (Fig. 5), which shows that the aglycone flavonoid naringenin is the precursor of hesperetin, naringin, neohesperidin and hesperidin. Naringin and neohesperidin are formed by the action of 1,2-rhamnosyltransferase on the 7-O-glucoside forms of naringenin and hesperetin, respectively; while hesperidin is generated by the action of 1,6-rhamnosyltranferase on hesperetin-7-O-glucoside. The high concentrations of naringin and neohesperidin in the bitter orange variety indicate a higher activity of 1,2-rhamnosyltranferase as compared to 1,6-rhamnosyltransferase. On the other hand, the rest glycoside flavonoids provided a behavior opposite to that observed for major flavonoids. Thus, fresh peels presented the highest concentration of glycoside flavonoids in overall terms, which were strongly decreased in lyophilized and oven-dried samples, except for homoorientin that was not affected by this behavior in the varieties Clemenules clementine and bitter orange. In these two varieties, this flavonoid was even increased in oven-dried peels of Clemenules clementine. The oven-dried peels were also characterized by high concentration of aglycone flavonoids, particularly, quercetin that reached a concentration around 500 ng/g in oven-dried peels in contrast to fresh and lyophilized peels in which this flavonoid was not found. Finally, the concentration of tangeretin decreased when the water content was removed from the peels of most varieties, being the effect more significant on oven-dried peels. Among the varieties following this behavior, it is worth mentioning the Sanguinelli orange peel due to its drastic decrease in tangeretin when samples were oven-dried, probably owing to degradation during the drying step. On the other hand, peels from Clemenules clementine, Navel Lane Late and bitter orange varieties reported a higher concentration of tangeretin when lyophilized, probably because lyophilization does not promote degradation of this polymethoxyflavone.

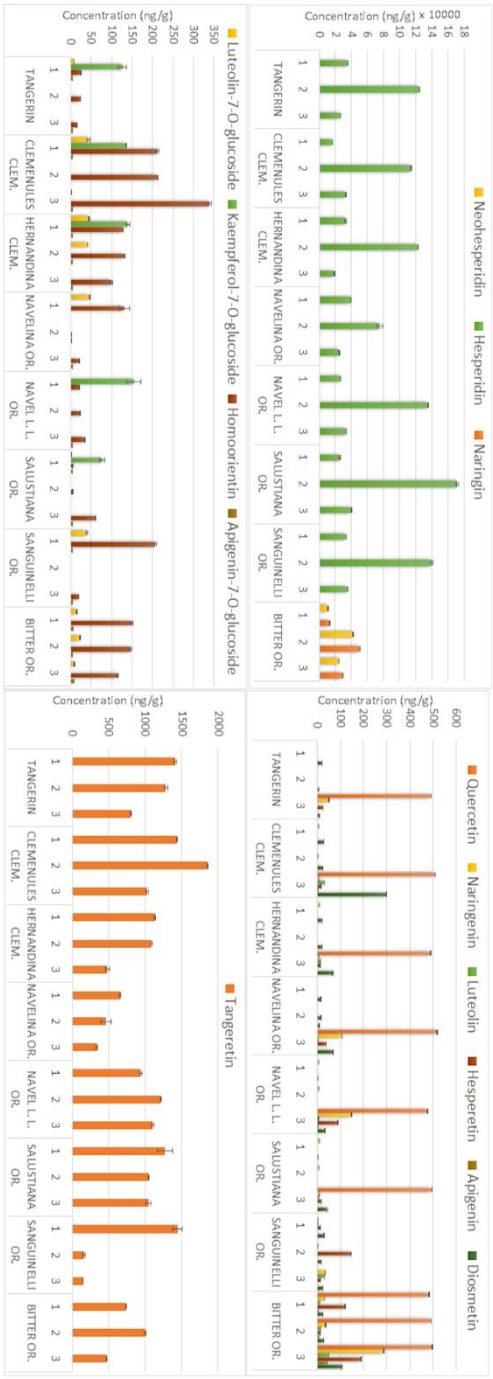

Figure 4. Concentration of flavonoids (as ng/g) grouped in four diagrams by their concentration level in the fresh (1), lyophilized (2) and oven-dried peels (3) of the different orange varieties.

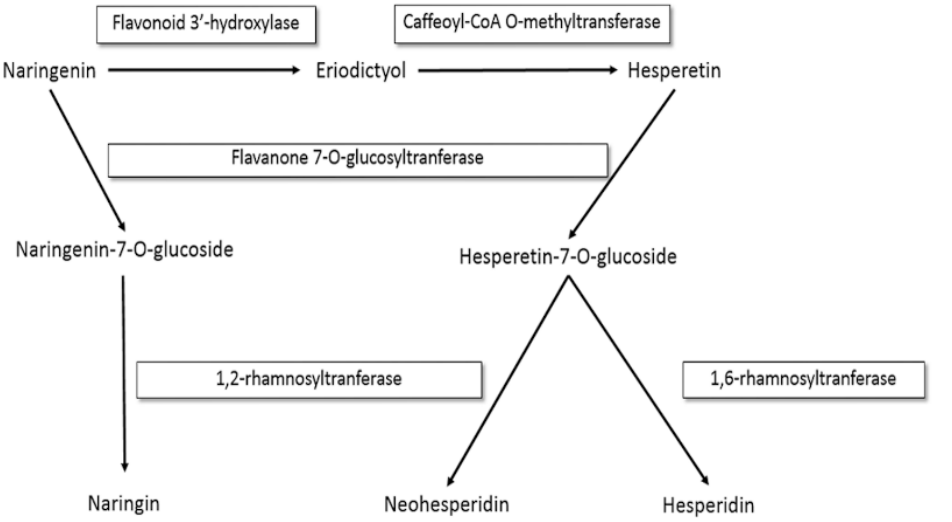

Figure 5. Pathways of the biosynthesis of hesperetin, naringin, neohesperidin and hesperidin.

4. Conclusions

An LC–MS/MS method was proposed for quantitation of 16 flavonoids with LODs and LOQs lower than those obtained in previous published research, thus improving the ability for determination of these compounds when they are present at low concentration. A simple extraction method was applied to obtain the target flavonoids from orange peels, demonstrating by a study of the extraction kinetics that the compounds are rapidly extracted from this matrix. Application of the proposed quantitation method to extracts of fresh orange peels evidenced that both the content in flavonoids is dependent on the variety of the orange and the water-removal step affects to the content of flavonoids in orange peels, although the intensity of this effect depends on the orange variety. Finally, the results obtained with the two methods for water removal allow concluding that lyophilization preserves the glycosides flavonoids, which are more concentrated in lyophilized peel; while oven-drying favors the formation of aglycone

flavonoids, probably owing to degradation of the glycosides forms by the drying temperature. Therefore, sample storage with water removal, when required, can be selected as a function of the subsequent use.

Acknowledgements

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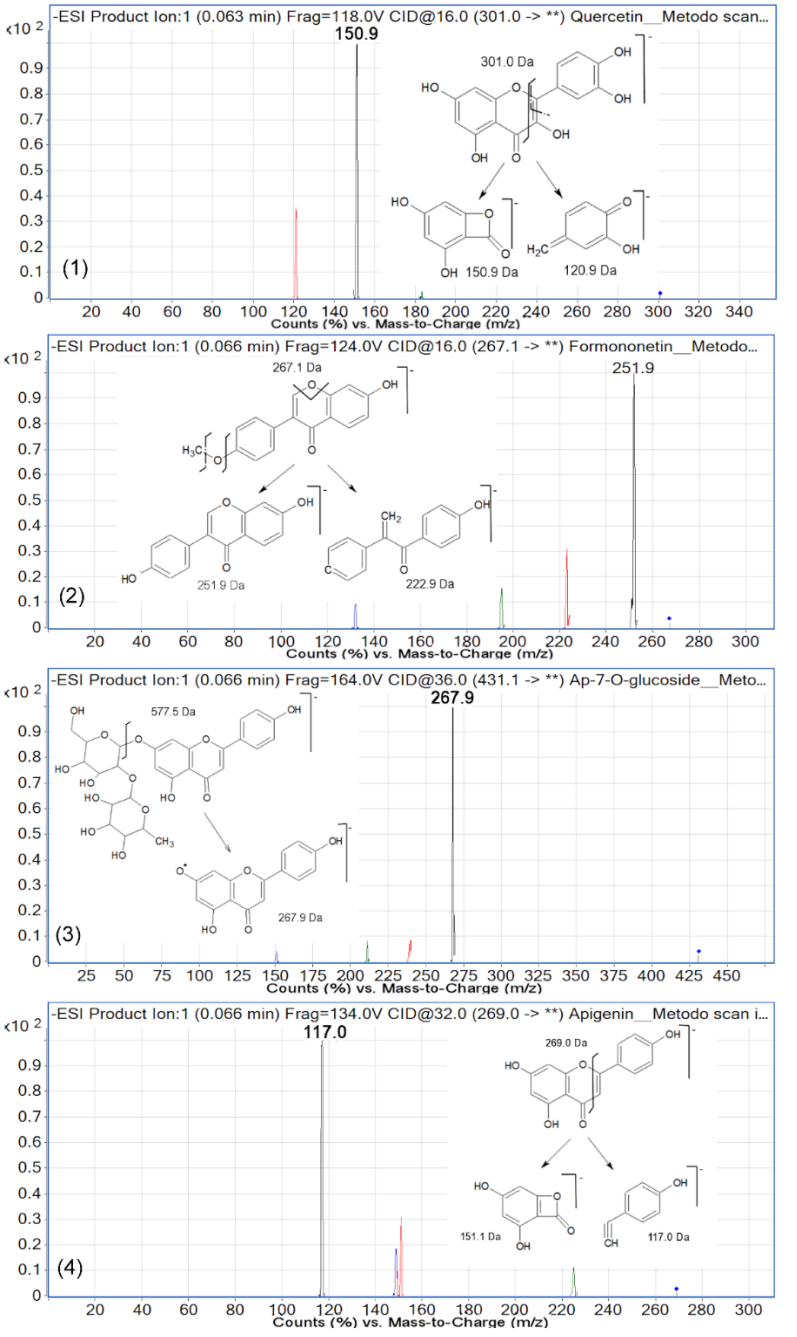
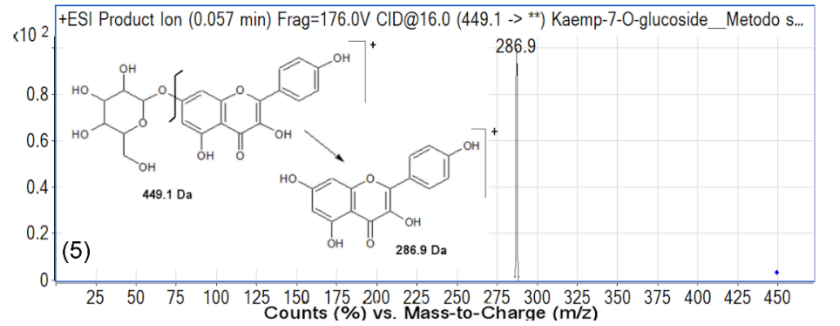
Supplementary Information

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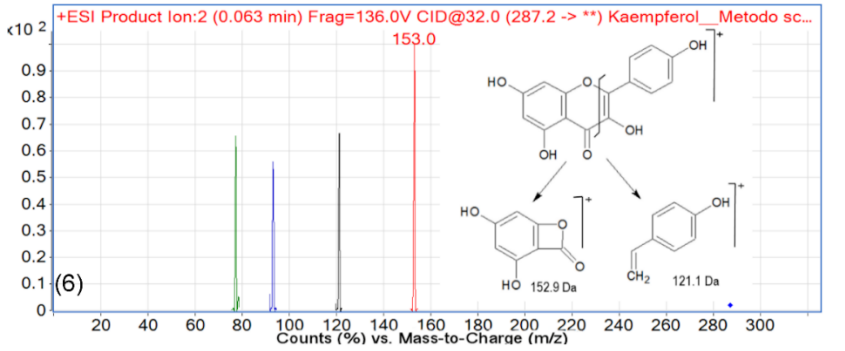
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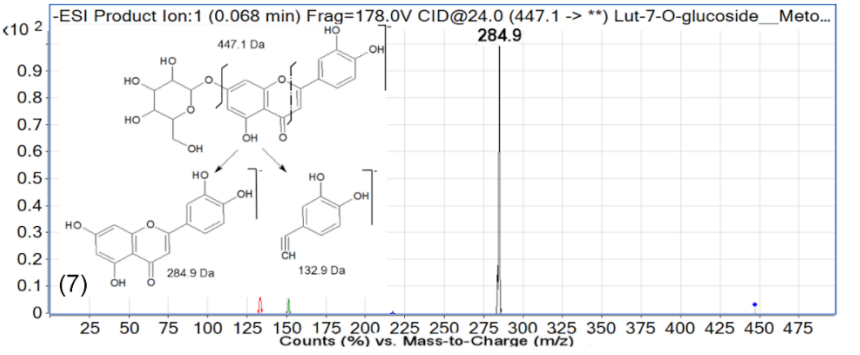
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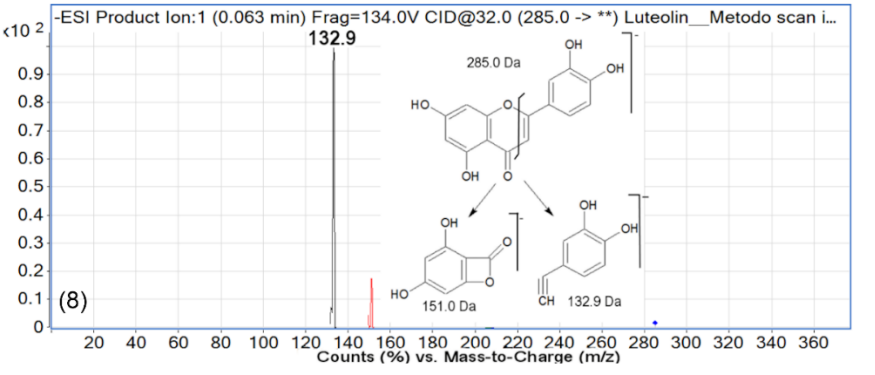
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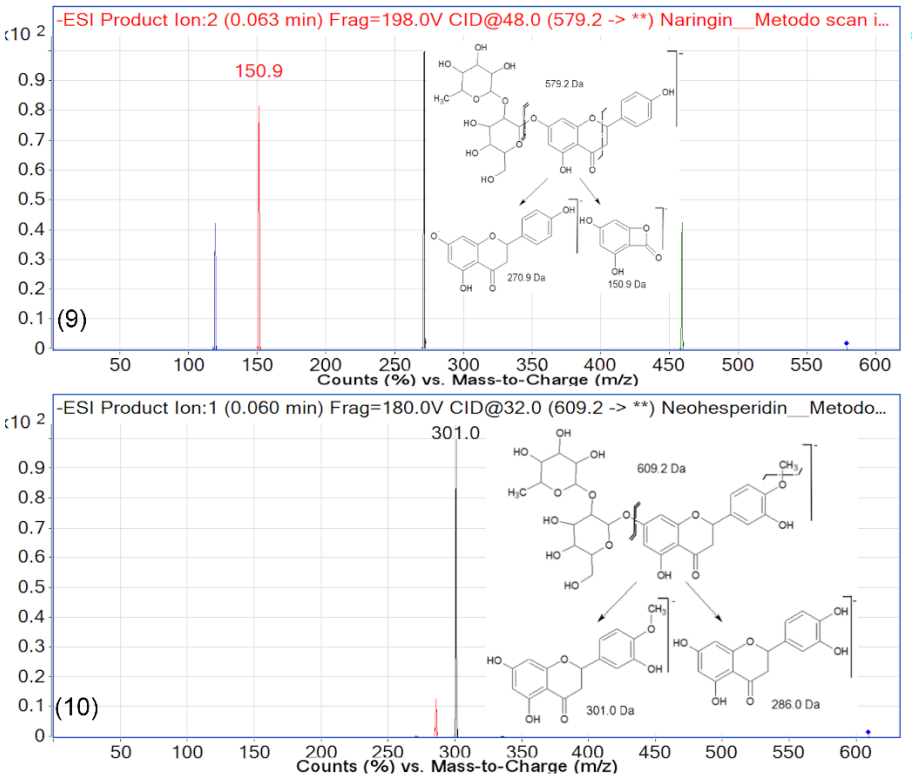
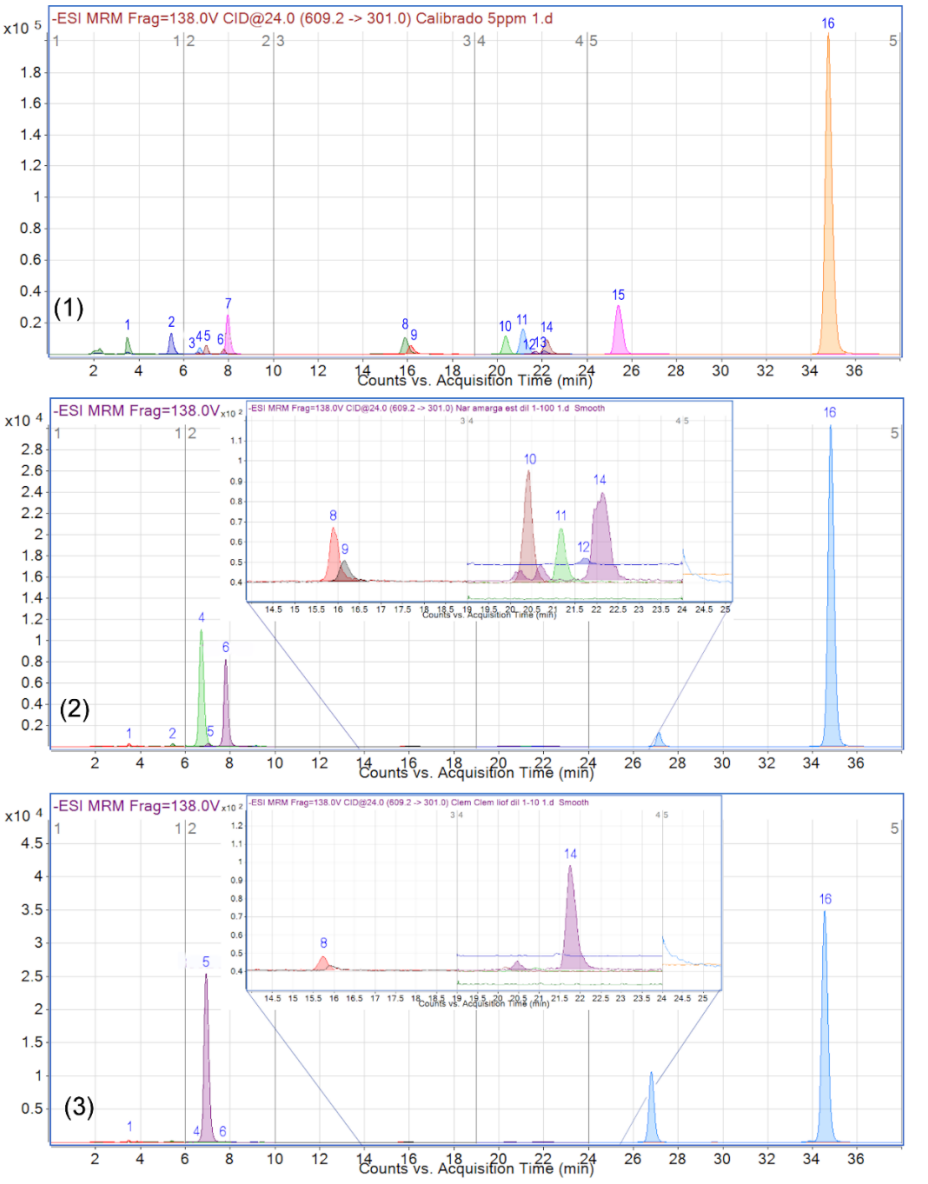
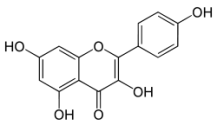
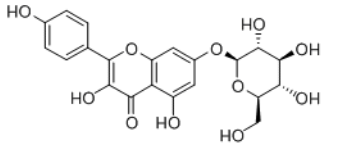
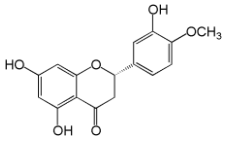
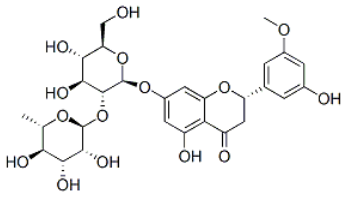
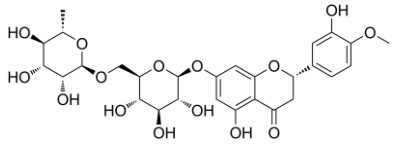
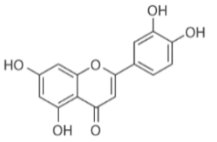
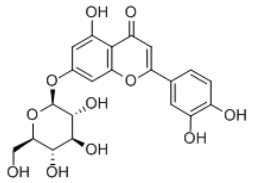
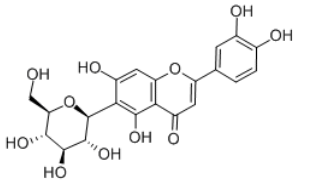


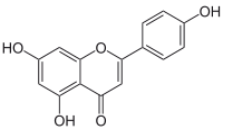
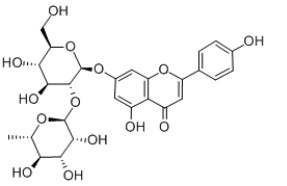
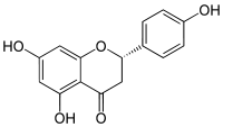
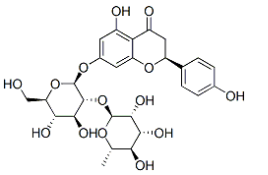
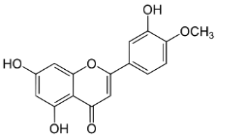
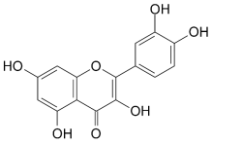
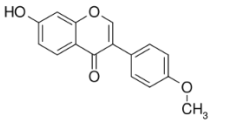
Supplementary Figure 1. MS/MS spectra and fragmentation patterns of quercetin (1), formononetin (2), apigenin-7-O-glucoside (3), apigenin (4), kaempferol-7-O-glucoside (5), kaempferol (6), luteolin-7-O-glucoside (7), luteolin (8), naringin (9) and neohesperidin (10).



Supplementary Figure 2. SRM chromatograms of target flavonoids from a multistandard solution (1), in the extract of oven-dried peel of bitter orange (2), and in the extract of lyophilized peel of clemenules clementine (3). 1, homoorientin; 2, luteolin-7-O-glucoside; 3, kaempferol-7-O-glucoside; 4, naringin; 5, hesperidin; 6, neohesperidin; 7, apigenin-7-O-glucoside; 8, luteolin; 9, quercetin; 10, naringenin; 11, apigenin; 12, hesperetin; 12, hesperetin; 13, kaempferol; 14, diosmetin; 15, formononetin; 16, tangeretin.

Supplementary Table 1. Classification of target analyzed flavonoids.

Flavonoid aglycone	Flavonoid glycoside
Kaempferol 	Kaempferol-7-O-glucoside 
Hesperetin 	Neohesperidin 
	Hesperidin 
Luteolin 	Luteolin-7-O-glucoside 
	Homoorientin 

Apigenin 	Apigenin-7-O-neohesperidoside 
Naringenin 	Naringin 
Diosmetin 	
Quercetin 	
Formononetin 	
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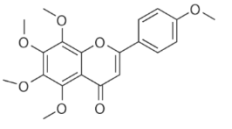
Tangeretin 	

PARTE B

Caracterización y cuantificación de metabolitos presentes en la hoja de estevia rebaudiana

Este segundo bloque de la Tesis abarca los estudios sobre estevia rebaudiana, que han permitido profundizar en una serie de aspectos analíticos de esta planta prácticamente desconocidos hasta ahora.

Un primer estudio no orientado de extractos polares y no polares de hojas de estevia rebaudiana, que se recoge en el Capítulo IV, se realizó con un equipo LC–QTOF MS/MS y proporcionó la identificación tentativa de 89 compuestos. Los compuestos identificados se clasificaron en diferentes familias, una de las cuales —la constituida por las amidas de ácidos grasos— fue identificada por primera vez. También se identificaron tentativamente nuevos esteviol glicósidos y se propusieron sus posibles estructuras.

Tras la información obtenida en el primer estudio global, los siguientes estudios se orientaron a ratificar la identificación y a desarrollar métodos —basados en el uso de equipos LC–QqQ— para la cuantificación en plantas de estevia rebaudiana de ciertas familias de compuestos. El objetivo final en este caso fue conocer las mejores condiciones de desarrollo de estas plantas para maximizar el contenido de las familias de interés.

El primer estudio cuantitativo se dedicó a los compuestos de mayor interés de la planta: los esteviósidos. Se utilizaron 8 patrones comerciales de estos compuestos con los que se optimizaron las condiciones de ionización y fragmentación de forma que se mejoraron los límites de detección y cuantificación obtenidos hasta la fecha: entre 0.1 y 0.5 ng/mL y entre 0.5 y 1.0 ng/mL, respectivamente. El método se aplicó a diferentes variedades de estevia desarrolladas en laboratorio, en invernadero y en campo, y se estudió la influencia en el contenido de esteviósidos cuando se utilizan sólo hojas o estas junto con las ramas. Este estudio, que constituye el Capítulo V, proporcionó pautas para el cultivo de estevia rebaudiana en Andalucía.

El Capítulo VI abarca el estudio cuantitativo de los compuestos fenólicos de

estevia rebaudiana, a los cuales se atribuyen las propiedades saludables de esta planta. En este caso se requirió un método dual para el análisis independiente de los ácidos cafeoilquínicos y los flavonoides debido a su diferente ionización. También en este caso se consiguieron límites por debajo de los ng/mL para la detección y cuantificación de estos compuestos. El método se aplicó a diferentes variedades genéticas de estevia para comparar su contenido fenólico.

CAPÍTULO IV

Characterization of Stevia leaves by LC–QTOF MS/MS analysis of polar and non-polar extracts

Food Chemistry

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Characterization of Stevia leaves by LC−QTOF MS/MS analysis of polar and non-polar extracts

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ABSTRACT

Stevia is currently a well-known plant thanks to its sweeting power. Numerous studies that elucidate its composition were exclusively focused on determination of steviol and its glycosides. Untargeted analysis was applied to obtain a profile of main compounds present in extracts from Stevia (*Stevia rebaudiana Bertoni*) leaves using LC−MS in high resolution mode with a quadrupole–time of flight analyzer. Eighty-nine compounds were tentatively identified and classified into different families: flavonoids, quinic and caffeic acids and derivatives, diterpenoids (including steviol and glycosides), sesquiterpenoids, amino acids and derivatives, fatty amides and derivatives, fatty acids and derivatives, oligosaccharides, glycerolipids, purines, and retinoids. New steviol glycosides were tentatively identified and their possible structures proposed. Other compounds were tentatively identified in Stevia for the first time, such as fatty acid amides. These results reveal the wide range of compounds present in Stevia, which could be responsible for the nutraceutical effects ascribed to their leaves.

Keywords Stevia leaves; Tentative identification; LC−QTOF; Comprehensive profile.

1. Introduction

Stevia (*Stevia rebaudiana Bertoni*) is a schrub of the Asteraceae family native to South America, especially to Northeast of Paraguay (Soejarto, 2002). Nowadays, the well-known sweet taste of Stevia leaves has turned this plant into a powerful alternative to artificial sweeteners and has spread Stevia crops to other regions of the world such as Canada, Asia and Europe (Lemus-Mondaca, Vega-Gálvez, Zura-Bravo, & Kong, 2012). The Stevia sweet taste is due to steviol glycosides such as rebaudioside A and stevioside as the most important, the wide research on which has promoted their approval as food additives in many countries worldwide. As an example, the European Food Safety Authority (EFSA) issued a positive scientific opinion on their safety and established their acceptable daily intake (Aguilar et al., 2010). In addition to their sweetening properties, some studies suggest that Stevia extracts are endowed with therapeutic properties as they possess recognized anti-hyperglycemic, anti-hypertensive, anti-inflammatory, anti-tumor, anti-diarrhea, diuretic and immunomodulatory effects (Chatsudthipong & Muanprasat, 2009). Furthermore, toxicological studies have shown that steviosides do not have mutagenic, teratogenic or carcinogenic effects, in contrast to artificial sweeteners (Pól, Hohnová, & Hyötyläinen, 2007).

The main body of the studies on Stevia has been focused on the identification and quantification of steviol glycosides, using Liquid Chromatography (LC) for separation and Mass Spectrometry (MS) for detection, in most cases. For example, Montoro et al. (2013) quantified six steviol glycosides in extracts from Stevia plants cultivated in different countries. Also, 8 steviol glycosides were quantified in our recent work by an optimized method based on LC−MS/MS with a triple quadrupole detector (Molina-Calle, Sánchez de Medina, Delgado de la Torre, Priego-Capote, & Luque de Castro, 2016).

The extensive knowledge achieved so far about some steviol glycosides contrasts with the lack of information about the composition of other families in

Stevia. Few Stevia researchers have focused their attention on other families different from steviol glycosides; among them, Karakose et al. studied the phenol composition of Stevia leaves (Karaköse, Jaiswal, & Kuhnert, 2011; Karaköse, Müller, & Kuhnert, 2015), and Periche, Koutsidis & Escriche (2014) quantified free amino acids in extracts from this plant. Wölwer-Rieck (2012) reviewed the literature on identification of compounds in Stevia and outlined its comprehensive profile; however, the overall profile has not been evaluated yet. Thus, the main objective of this study was to characterize Stevia leaves by analysis of extracts obtained with polar and non-polar solvents in order to increase the detection coverage.

2. Materials and methods

2.1. Samples and reagents

The Stevia (*Stevia rebaudiana Bertoni*) leaves were provided by Vitrosur S.L. in 2014 from several plants (n=10) selected randomly in a plot of land in Lebrija (Seville, Spain) that were grown for four months. The leaves were collected during the flowering of the plant in September; then, they were oven-dried at 30 ^{o}C for 24 h and milled using a domestic grinder to obtain a homogeneous particle size. After homogenization, the samples were taken.

All solvents were LC grade or higher. Deionized water (18 MΩ·cm) was from a Millipore (Bedfore, MA, USA) Milli-Q plus system. Methanol, ethanol and *n*-hexane were from Scharlab (Barcelona, Spain).

2.2. Extraction procedure

The extraction was carried out by maceration of 0.5 g of Stevia leaves for 2 h using 50 ml of a 35:65 (v/v) ethanol−water mixture or *n*-hexane for extraction of polar and non-polar compounds, respectively. An aliquot of the polar extract was

1/100 diluted prior to analysis, while no dilution of the non-polar extract was required.

2.3. LC−QTOF MS/MS analysis

An Agilent 1200 LC coupled to a quadrupole–time of flight (QTOF) 6540 UHD accurate mass spectrometer (MS) was used to tentatively identify the metabolites in the extracts. The analytical column was a Mediterranea Sea C18 analytical column (5 µm, 15 × 0.46 cm) from Teknokroma (Barcelona, Spain). The mobile phases were deionized water with 0.1% of formic acid (A) and methanol with 0.1% of formic acid (B) and the programmed gradient was as follows: start with 10% B, change from 10% to 100% B in 25 min and constant 100% B for 10 min more. A post-run of 5 min was programed to equilibrate the column between analyses. The constant flow was 0.7 ml/min and 10 µl of sample was injected from the autosampler thermostated at 5 ^{o}C. Dual electrospray ionization (ESI) source and mass spectrometer parameters, operating in negative and positive ionization modes, were as follows: capillary, skimmer, and Q1 voltage were set at 3500, 65, and 130 V, respectively; N_2 nebulizer gas was flowed at 40 psi; N_2 drying gas flow rate and temperature were 10 l/min and 350 °C; N_2 sheath gas flow rate and temperature were 10 l/min and 325 ^{o}C; and octopole radiofrequency voltage was set at 750 V. The extracts were analyzed in positive and negative ionization modes at two values of collision energy, 20 and 40 eV, using the centroid mode. The extracts were analyzed in auto MS/MS mode (*m/z* range 40−2000) and the data collected at five spectra per second in the extended dynamic range mode. The auto MS/MS mode was configured with 2 maximum precursors per cycle and an exclusion window of 1 min after two consecutive selections of the same precursor. To assure the desired mass accuracy of the recorded ions, continuous internal calibration was performed during analyses with the use of signals at *m/z* 121.0509 (protonated purine) and *m/z* 922.0098 [protonated hexakis (1H, 1H, 3H-tetrafluoropropoxy)phosphazine or HP-921] in the positive ionization mode; and

m/z 112.9856 (trifluoroacetic acid anion) and *m/z* 1033.9881 (HP-921) in the negative ionization mode.

2.4. Data treatment

Mass Hunter Workstation software (version B 07.00 Qualitative Analysis, Agilent Technologies, Santa Clara, CA, USA) was used to extract potential molecular features (MFs) from all the data sets. The extraction algorithm considered all ions exceeding 400 counts with a single charge. The isotopic distribution for inclusion of MFs should be defined by two or more ions (with a peak spacing tolerance of *m/z* 0.0025 and 10.0 ppm in mass accuracy). Adducts formation in positive (+H, +Na, +K, $+NH_4$) and negative ionization (-H, +Cl) modes, as well as neutral loss by dehydration, were included to identify MFs corresponding to the same potential metabolite. The raw data were filtered with a minimum counts level of 5000 for samples analyzed in the negative ionization mode and 3000 in the positive mode. The resulting MFs were tentatively identified by searching MS and MS/MS information on different databases such as METLIN (http://metlin.scripps.edu/), Food Database (http://foodb.ca/), MassBank (http://www.massbank.jp/) and MetFrag (http://msbi.ipb-halle.de/MetFrag/). The selected precursor ions for identification were +H, +Na and $+H\text{-}H_2O$ adducts for positive precursor ions and –H and +Cl adducts for negative precursor ions, establishing an error limit of 4 ppm for identification.

3. Results and discussion

3.1. Tentative identification of compounds present in Stevia leaves

Polar and non-polar extracts of Stevia leaves were analyzed by LC−QTOF MS/MS as described in the materials and methods section. Fig. 1 shows the MS total ion chromatograms (TIC) provided by analysis of the polar extract in the positive and negative ionization modes. As can be seen, several groups of peaks

were eluted along the chromatogram in both ionization modes, which gives an idea of different families of compounds in the extracts. On the other hand, the non-polar extract was clearly much poorer in composition than the polar extract since only the last part of the chromatogram presented chromatographic peaks (see Supplementary Fig. 1). Tentative identification of MFs extracted as described in the Experimental section using MS/MS data in high resolution mode led to a total number of 89 compounds with an accuracy error below 4 ppm. The tentatively identified compounds classified by families and the main parameters that support their identification are listed in Table 1. Most of the compounds were identified in the polar extract, being phenolic compounds, terpenoids and amino acids the main families in this extract. On the other hand, only glycerolipids were exclusively identified in the non-polar extract. Other families whose compounds are characterized by a medium polarity, such as fatty acids and amides and derivatives, were detected in both extracts.

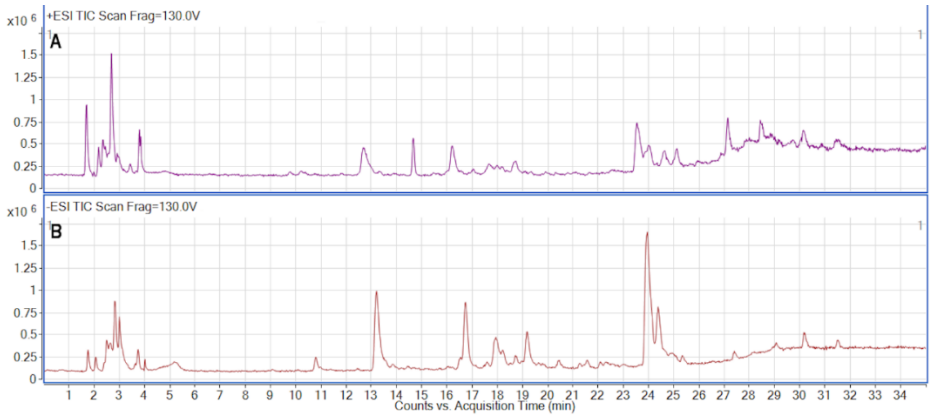

Figure 1. MS total ion chromatograms from the polar extract in positive (A) and negative (B) ionization modes.

Table 1. Tentatively identified compounds in the polar and non-polar extracts from Stevia leaves and the main parameters that support their identification.

Family	Compound	Mass (Da)	Retention time (min)	Polarity	Adduct	*m/z*	Fragments	Error	Previously reported by
Phenolic compounds									
Flavonoids	Kaempferol monoglycoside	418.0897	19.65	−	M−H	417.0824	255.0254, 284.0292	0	Karakose et al., 2015
	Kaempferol rhamnoside	432.1055	20.44	−	M−H	431.0982	227.0313, 255.0263, 284.0287	0	
	Flavonoid monoglycoside	434.0848	18.99	−	M−H	433.0775	255.0263, 271.0213, 300.0240	0	
	Quercetin-3-O-arabinoside	434.0849	18.75	−	M−H	433.0776	255.0260, 271.0210, 300.0235	0	
	Quercitrin	448.0997	19.18	−	M−H	447.0924	255.0260, 271.0210, 300.0235	1	Karakose et al., 2015
	Flavonoid monoglycoside	448.1002	17.61	−	M−H	447.0929	285.0367	0	
	Flavonoid diglycoside	594.1579	18.97	−	M−H	593.1506	255.0269, 284.0291	1	
	Rutin	610.1534	18.24	−	M−H	609.1461	151.0001, 300.0243	0	Karakose et al., 2015
	Flavonoid coumaroylglucoside-glucoside	756.1907	19.84	−	M−H	755.1834	469.1316, 593.1479		
	Quercetin 3-O-(coumaroylglucoside)-7-O-glucoside	772.1857	19.23	−	M−H	771.1784	301.0315, 609.1441	0	
Quinic and caffeic acid and derivatives	Quinic acid	192.0624	3.02	−	M−H	191.0551	59.0117, 85.0268, 93.0324	4	
	Quinate phosphate	290.0403	3.04	−	M−H	289.0330	191.0526		
	4-Caffeoylquinic acid	354.0918	10.88	−	M−H	353.0845	85.0270, 135.0422, 191.0526	1	Karakose et al., 2011, Karakose et al., 2015
	5-Caffeoylquinic acid	354.0918	13.14	−	M−H	353.0845	85.0270, 135.0422, 191.0526	1	Karakose et al., 2011, Karakose et al., 2015
	3-Caffeoylquinic acid	354.0918	13.86	−	M−H	353.0845	59.0119, 85.0270, 191.0526	1	Karakose et al., 2011, Karakose et al., 2015

Cont. Table 1

	1,5-Dicaffeoylquinic acid	516.1238	16.72	−	M−H	515.1165	179.0316, 191.0527, 353.0847	2	Karakose et al., 2011, Karakose et al., 2015
	3,4-Dicaffeoylquinic acid	516.1238	19.13	−	M−H	515.1165	179.0316, 191.0527, 353.0847	2	Karakose et al., 2011, Karakose et al., 2015
	4,5-Dicaffeoylquinic acid	516.1238	17.91	−	M−H	515.1165	179.0316, 191.0527, 353.0847	2	Karakose et al., 2011, Karakose et al., 2015
	Caffeic acid	180.0422	2.537	−	M−H	163.0389	59.0115, 87.0059, 161.0430	3	
Terpenoids									
Diterpenoids	Austroinulin	322.2502	27.51	−	M+Cl	357.2196	140.4959, 158.9226, 271.2262	1	Yoda, Marquues, Petenate, & Meireles, 2003; Ibrahim, El-Gnegaihi, Motawe, & Riad, 2007
	Steviol	318.2200	23.57	+	M+H	319.2273	255.2112, 273.2208, 301.2141	0	
	Steviolbioside	642.3233	23.95	−	M−H	641.3160	479.2616	0	Montoro et al., 2013
	Rubusoside	642.3241	25.35	−	M−H	641.3168	479.262	1	Espinoza et al., 2014
	Steviol+Glucose+4-Methylglucuronide	688.3299	24.89	−	M−H	687.3226	479.2514, 641.3150		
	Dulcoside A	788.3827	24.41	−	M−H	787.3754	479.2624, 625.3202	0	Espinoza et al., 2014; Periche, Castelló, Heredia, & Escriche, 2015
	Stevioside	804.3769	23.95	−	M−H	803.3696	479.2597, 641.3150	1	Espinoza et al., 2014; Montoro et al., 2013; Periche, Castelló, Heredia, & Escriche, 2015
	Steviol+2 Glucoses+ 4-Methylglucuronide	850.3829	23.95	−	M−H	849.3756	641.3160, 803.3688		
	Steviol+2 Glucoses+2 Xyloses	907.4057	23.87	+	M+H	908.4130	319.2266, 411.1139, 567.2791		
	Rebaudioside A	966.4325	23.97	−	M−H	965.4252	803.3689	1	Espinoza et al., 2014; Montoro et al., 2013; Periche, Castelló, Heredia, & Escriche, 2015

Cont. Table 1

	Rebaudioside B	804.3787	24.69	−	M−H	803.3714	641.3162	0	Espinoza et al., 2014; Montoro et al., 2013
	Rebaudioside C	950.4354	24.38	−	M−H	949.4281	787.3749	0	Espinoza et al., 2014; Montoro et al., 2013; Periche, Castelló, Heredia, & Escriche, 2015
	Rebaudioside D	1128.4839	22.10	−	M−H	1127.4766	803.3738	1	Espinoza et al., 2014
	Rebaudioside E	966.4292	21.59	−	M−H	965.4219	641.3144, 805.9837	1	
	Rebaudioside F	936.4207	24.27	−	M−H	935.4134	773.3584	0	Espinoza et al., 2014
	Dulcoside A derivative	990.4297	24.48	−	M+FA−H	1035.4279	787.3736		
	Stevioside derivative	1052.4315	24.10	−	M−H	1051.4242	803.3678, 1007.4329		
Sesquiterpenoids	Sterebin I/J	336.2295	24.51	−	M+FA−H	381.2277		2	McGarvey et al., 2013
	Sterebin I/J	336.2295	24.92	−	M+FA−H	381.2277		2	McGarvey et al., 2013
	Sterebin E/F/M/N	338.2453	25.01	−	M+FA−H	383.2435		1	Oshima, Saito, & Hikino, 1998; McGarvey et al., 2003; Ibrahim et al., 2007
	Sterebin E/F/M/N	338.2453	24.75	−	M+FA−H	383.2435		1	
	Sterebin E/F/M/N	338.2453	26.46	−	M+FA−H	383.2435		1	
	Sterebin E/F/M/N	338.2453	25.69	−	M+FA−H	383.2435		1	
Other families									
Amino acids and derivatives	Aminobutyric acid	103.0638	2.13	+	M+H	104.0711	60.0809, 69.0334, 87.0385	4	
	Serine	105.0429	2.35	+	M+H	106.0502	44.9965, 60.0450	3	Periche et al., 2014
	Proline	115.0633	2.69	+	M+H	116.0706	70.0648	4	Periche et al., 2014
	Pyroglutamic acid	129.0422	2.43	+	M+NH4	147.0760	56.0495, 84.0440	2	
	Choline	103.0997	2.19	+	M+H	104.1070	44.0494, 45.0334, 58.0653	2	
	Alanine	89.0477	2.39	+	M+H	90.0550	44.0495, 55.9336	1	Periche et al., 2014
	Asparagine	132.0545	2.37	+	M+H	133.0608	46.0283, 74.0239, 87.0554	0	Periche et al., 2014
	Glutamate	147.0531	2.49	+	M+H	148.0604	56.0490, 84.0444, 130.0488	0	Periche et al., 2014

Cont. Table 1

	Leucine/Isoleucine*	131.0946	2.86	+	M+H	132.1019	44.0408, 69.0596, 86.0952	0	Periche et al., 2014; Esmat Abou-Arab, Azza Abou-Arab, & Ferial Abu-Salem, 2010
	Lysine*	146.1055	1.99	+	M+H	147.1128	67.0542, 84.0804, 101.1073	0	Esmat Abou-Arab, Azza Abou-Arab, & Ferial Abu-Salem, 2010
	Threonine*	119.0582	2.42	+	M+H	120.0655	56.0492, 74.0593,	0	Esmat Abou-Arab, Azza Abou-Arab, & Ferial Abu-Salem, 2010
	Tryptophan*	204.0899	9.70	+	M+H	205.0972	146.0598, 159.0917, 170.0586	0	
	Valine*	117.0790	2.96	+	M+H	118.0863	56.0545, 72.0810	1	Periche et al., 2014; Esmat Abou-Arab, Azza Abou-Arab, & Ferial Abu-Salem, 2010
Fatty acid amides and derivatives	Palmitamide	255.2554	29.06	+	M+H	256.2627	57.0695, 88.0745, 102.0910	3	
	Oleamide	281.2711	29.22	+	M+H	282.2784	57.0696, 100.0761, 111.1163	2	
	Estearamide	283.2867	30.20	+	M+H	284.2940	57.0695, 88.0745, 102.0910	3	
	13-Docosenamide	337.3341	31.53	+	M+Na	360.3233	55.0540, 69.0699, 83.0857	1	
	N-stearoyl valine	383.3340	29.74	+	M+Na	406.3292	57.0699, 69.0699, 83.0856	1	
Fatty acids and derivatives	Myristic acid	228.2094	29.16	−	M−H	227.2021	68.9934, 121.9820	1	
	Palmitic acid	256.2399	30.25	−	M−H	255.2326	78.9564, 158.9204	1	
	Stearic acid	284.2679	31.58	−	M−H	283.2636	68.9933, 78.9564, 152.6406	2	
	Oleic acid	282.2558	29.07	−	M+FA−H	327.2540	44.9960, 112.9821, 183.0092	1	
	Oleic acid derivative	401.3512	23.70	+	M+H	402.3585	57.0699, 71.0855, 283.2630		
	Oleic acid derivative	402.2382	25.14	+	M+H	403.2455	283.2626, 343.2204, 361.2349		
	Gondoic acid derivative	429.3820	24.62	+	M+H	430.3893	57.0699, 311.2940		

Cont. Table 1

Glycerolipids	MG(16:1)	328.2614	28.87	+	M+Na	353.2661		2	
	MG(16:0)	330.2770	29.15	+	M+Na	351.2506		0	
	MG(18:1)	356.2927	22.52	+	M+NH4	374.3261		3	
	MG(18:0)	358.3083	30.24	+	M+Na	381.2976		1	
	MG(20:1)	384.3242	23.72	+	M+NH4	402.3580		1	
	MG(22:1)	412.3555	24.65	+	M+NH4	430.3893		0	
	MG(22:2)	410.3398	23.23	+	M+NH4	428.3739		1	
	DG(18:1/0:0/20:1)	650.5858	27.54	+	M+NH4	668.6196		1	
	DG(18:0/0:0/18:1)	622.5560	26.87	+	M+NH4	640.5901		3	
	DG(18:1/0:0/16:0)	594.5227	26.53	+	M+NH4	612.5565		1	
	DG(18:1/0:0/18:1)	620.5376	26.59	+	M+NH4	638.5717		1	
	DG(18:1/0:0/20:0)	650.5856	27.15	+	M+NH4	668.6196		1	
	DG(18:2/0:0/20:0)	648.5699	26.91	+	M+NH4	666.6039		1	
	DG(20:0/0:0/20:1)	678.7163	27.42	+	M+NH4	696.6512		2	
	DG(20:1/0:0/20:2)	674.5861	26.97	+	M+NH4	692.6191		1	
	TG(16:1/20:0/20:0)	916.8460	28.33	+	M+NH4	934.8803		0	
Oligosaccharides	Gamma-cyclodextrin	972.3163	23.57	+	M+3H	325.1127	85.0284, 127.0391, 145.0496	0	
	Disaccharide	359.1431	2.73	+	M+H	360.1504	69.0333, 85.0285, 127.0385	1	
	Trehalose	388.1222	2.86	−	M−H	387.1149	59.0116, 71.0116, 89.0217	1	
	Maltose+phosphate	440.0925	2.92	−	M−H	439.0852	78.9567, 96.9671		
Purines	Purine	120.0436	3.79	+	M+H	121.0509	51.0122, 65.0270, 77.0264	0	
Retinoids	Retinol derivative		27.21	+	M+H		69.0701, 81.0696, 119.0858		

3.2. Phenolic compounds

Phenols are metabolites widely present in the plant kingdom that are well known thanks to their beneficial properties to the organism, as recently reviewed by Roleira et al. (2015). Several studies have focused on phenols composition in Stevia plants; however, most of them involved overall determination by simple methods as those based on derivatization with the Folin-Ciocalteu reagent, their ferric reducing antioxidant power (FRAP), their reaction with 2,2-diphenyl-1-picrylhydrazyl (DPPH) or their radical scavenging activity (Gawel-Bęben et al., 2015; Kim, Yang, Lee, & Kang, 2011; Periche et al., 2014; Shukla, Mehta, Mehta, & Bajpai, 2012; Tadhani, Patel, & Subhash, 2007; Tavarini & Angelini, 2013; Zeng, Cai, Yang, & Wu, 2013). These studies only provided data of the total concentration of phenols and they did not report information about the nature and concentration of individual compounds. The most comprehensive study on phenolic compounds in Stevia leaves was carried out by Karaköse et al. (2011), who characterized and quantified 24 hydroxycinnamate derivatives in methanolic extracts using LC−MS^n with an ion-trap spectrometer. A recent study from the same authors analyzed 15 flavonoids also in methanolic extracts from Stevia leaves using the same methodology (Karaköse et al., 2015).

In the research reported here 21 phenolic compounds were tentatively identified in the polar extracts from Stevia leaves, which were classified in two main families: flavonoids, and quinic and caffeic acids and derivatives. All them were identified with high accuracy by the negative ionization mode, as the $[M-H]^-$ adduct form. The identification of flavonoids, located within the chromatographic retention interval 18–21 min, was complicated because most of them are isomers and their identification is unfeasible without comparison with MS/MS spectra in the databases. Thus, only isomers for which MS/MS spectra exist in the consulted databases could be identified. Among them, quercetin derivatives provided the highest chromatographic peaks, being quercitrin (*m/z* 447.0924) among the 10 most

intense peaks in the polar extract. Fig. 2 shows the extracted-ion chromatogram and product ion spectra of quercitrin and other two quercetin derivatives identified in the extract: rutin (*m/z* 609.1461) and quercetin-3-O-arabinoside (*m/z* 433.0776). The product ion spectra of the three flavonoids show that all of them are characterized by the *m/z* 300.02 fragment, which fits the aglycon moiety. Also a fourth quercetin derivative, quercetin-3-O-(coumaroylglucoside)-7-O-glucoside, was tentatively identified, but this peak was much less intense than those of the previous compounds. Finally, two kaempferol flavonoids were tentatively identified, one of them as kaempferol rhamnoside (*m/z* 431.0982), but the bonding position of the glycoside could not be determined, while the other could only be identified as a monoglycoside derivative (*m/z* 433.0775).

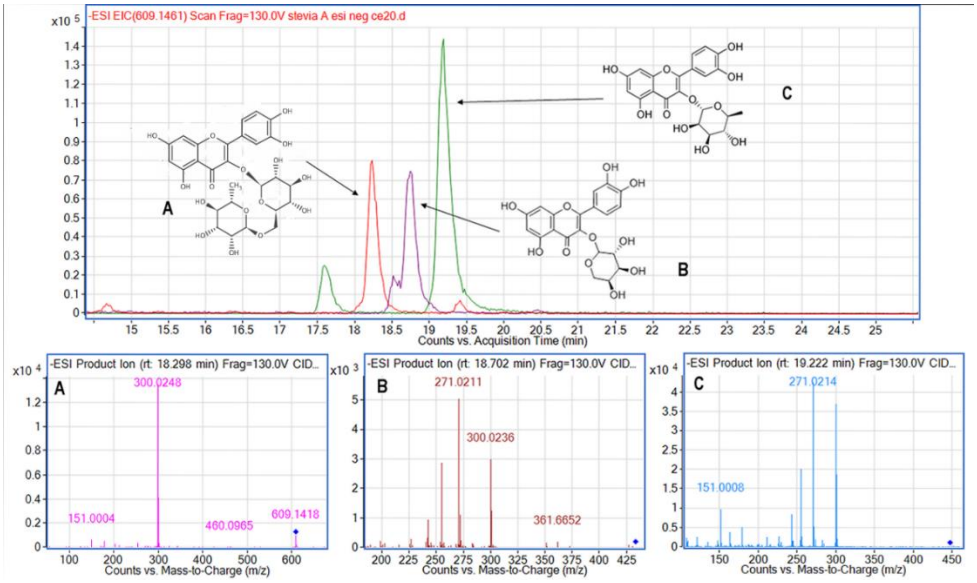

Figure 2. Extracted ion chromatograms and MS/MS spectra of rutin (A), quercetin-3-O-arabinoside (B) and quercitrin (C).

The family that encompasses quinic and caffeic acids and derivatives was also remarkable in the polar extracts due to the number of identified compounds. The most important compounds in this family were mono- and dicaffeoylquinic

acids, constituted by quinic acid bonded to one or two caffeic moieties at different positions. Several authors have studied the effect in the organism of these compounds extracted from leaves of different plants, and to which antimutagenic (Yoshimoto et al., 2002), neuroprotective (Nakajima, Shimazawa, Mishima, & Hara, 2007), antiviral (Li, But, & Ooi, 2005), and antibacterial properties (Jin et al., 2014) were attributed. However, only in the previously mentioned works by Karaköse et al. (2011, 2015) caffeoylquinic acids were studied in Stevia leaves, determining 14 of these compounds. In this study, 9 caffeoylquinic acids were detected in the chromatographic interval 10–19 min, excluding caffeic, quinic acid, and quinic phosphate, which appeared at min 3 due to their higher polarity. As can be seen in Fig. 3, three chromatographic peaks were provided from each, the mono- and dicaffeoylquinic acids, the extraction of each $[M-H]^-$ ion being *m/z* 353.0845 and *m/z* 515.1165, respectively. Additionally, the spectra recorded at 40 V of collision energy from mono- and dicaffeoylquinic acids show that both compounds gave the same most intense fragment at *m/z* 191.0531, which corresponds to the quinic acid residue. The peaks were assigned to the corresponding isomer by comparing the elution time and the obtained fragmentation pattern with those reported by Su et al. (2014). Supplementary Fig. 2 shows the fragmentation pattern of the mono- and dicaffeoylquinic isomers. The spectra used for comparison among isomers were those recorded at 20 V of collision energy since a slight fragmentation level allowed a better discrimination based on the intensity of some of the fragments. The three isomers of monocaffeoylquinic acid were identified by the intensity of the *m/z* 179.0316 and 135.0422 fragments, which were clearly more intense for the 4-caffeoylquinic acid. Finally, all dicaffeoylquinic acid isomers yielded the *m/z* 353.0844 fragment, corresponding to the loss of a caffeic residue as the most intense ion, although they could be distinguished thanks to the different intensity of the *m/z* 173.0422, *m/z* 179.0317, and *m/z* 191.0531 fragments.

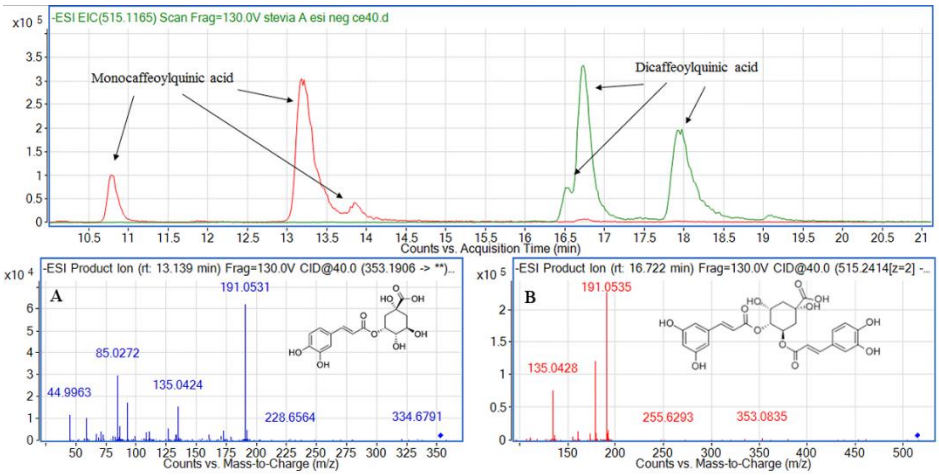

Figure 3. Extracted ion chromatograms of the monocaffeoylquinic acid (A) and dicaffeoylquinic acids (B) and the MS/MS spectrum recorded at 40 V of collision energy of the most intense peak from each one.

3.3. Terpenoids

Terpenoid metabolites tentatively identified in Stevia extracts were classified into two main families: diterpenoids, which essentially included steviol and their glycosides, and sesquiterpenoids. Apart from steviol and steviosides, austroinulin was also identified in the diterpenoids family, which was previously reported in Stevia leaves by Yoda, Marques, Petenate, & Meireles (2003) and Ibrahim, El-Gengaihi, Motawe, & Riad (2007). Anti-inflamatory effects have been attributed to austroinulin by Cho et al. (2013) through the inhibition of the production of proinflamatory cytokines. In this research, austroinulin was identified both in the polar and non-polar extracts of Stevia leaves due to its intermediate polarity. This diterpenoid was detected in the negative ionization mode by formation of the $[M+Cl]^-$ adduct.

Within the family of steviol and its glycosides, 16 compounds were tentatively identified in the polar extract of Stevia leaves. These compounds were found in the chromatographic interval 21–25 min by the negative ionization mode,

leading to the $[M-H]^-$ adduct —excluding steviol and steviol + 2 glucoses + 2 xyloses detected in the positive ionization mode and generating the $[M+H]^+$ adduct. Some of the steviol glycosides have been widely studied, as shown in the literature, mainly quantified in Stevia extracts using a mass detector (Espinoza et al., 2014; Montoro et al., 2013; Periche, Castelló, Heredia, & Escriche, 2015). In our previous study the fragmentation pattern of stevioside, steviolbioside, rubusoside, dulcoside A and rebaudiosides A−D was evaluated. This allowed confirming the presence of the most common steviol glycosides reported in the literature (Molina-Calle, Sánchez de Medina, Delgado de la Torre, Priego-Capote, & Luque de Castro, 2016). Additionally, the structures of other steviol glycosides were tentatively elucidated in the research proposed here. Two of the most interesting were identified as steviol + glucose + 4-methylglucuronide —named as 13-[(4-methylglucuronyl)oxy]kaur-16-en-18-oic acid 2-D-glucopyranosyl ester— and steviol + 2 glucoses + 4-methylglucuronide —named as 13-[(4-methylglucuronyl)oxy]kaur-16-en-18-oic acid 2-D-glucopyranosyl-D-glucopyranosyl ester. Fig. 4 shows the extracted ion chromatogram of these compounds and their tentatively proposed structure. The elution order for both compounds is in agreement with that provided for other steviol glycosides identified in the same extract. Thus, rebaudioside E and stevioside are a diglycoside and a monoglycoside, respectively, conjugated at the same position as the chemical structures here proposed for 4-methylglucuronide derivatives. Due to this additional glucose, rebaudioside E eluted at a retention time about 1 min shorter than that of stevioside (see Table 1). The same retention time shift was observed for steviol + glucose + 4-methylglucuronide and steviol + 2 glucoses + 4-methylglucuronide (see Fig. 4). One other alternative to explain the elution order would be glucose conjugation to the hydroxyl group of steviol. However, this alternative was discarded because in this case the elution time would be the same for the two compounds, as is the case with stevioside and rebaudioside A. For this reason, the two structures could be proposed (see Supplementary Fig. 3).

Concerning the MS/MS spectra of precursor ions for steviol + glucose + 4-methylglucuronide and steviol + 2 glucoses + 4-methylglucuronide, they were dominated by two main fragments (see Fig. 4). In the former case (steviol + glucose + 4-methylglucuronide) the *m/z* 641.3164 fragment corresponded to the loss of the carboxyl group in the 4-methylglucuronide moiety; while the fragment *m/z* 479.2619 was generated by the loss of the 4-methylglucuronide moiety. The same losses occurred in steviol + 2 glucoses + 4-methylglucuronide, which led to the fragments *m/z* 803.3673 and *m/z* 641.3156, respectively. This behavior indicates that the 4-methyglucuronide moiety was not joined to the additional glucose, the latter being joined to the other glucose moiety.

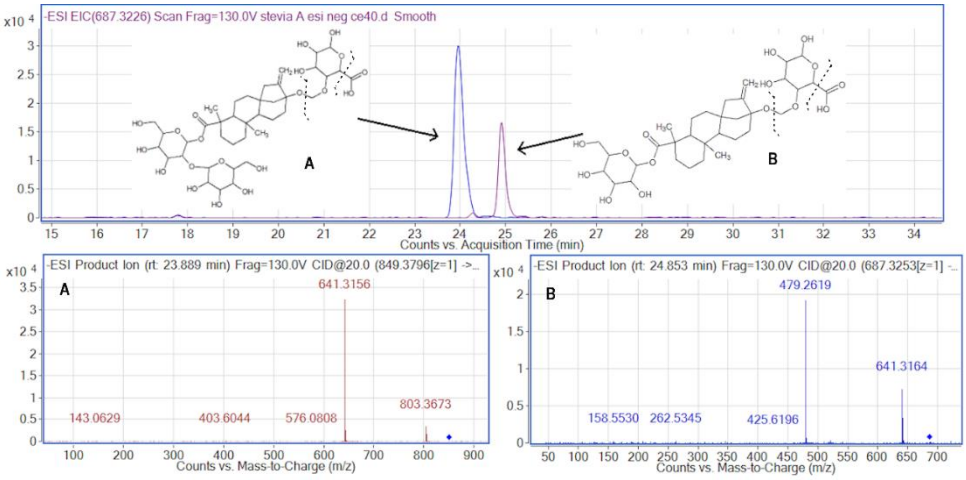

Figure 4. Extracted ion chromatogram of the steviol + glucose + 4-methylglucuronide (A) and steviol + 2 glucoses + 4-methylglucuronide (B), the structure proposed for each one and its MS/MS spectrum.

Concerning sesquiterpenoids, five sterebin isomers were tentatively identified in both the polar and non-polar extracts. These compounds eluted in the chromatographic interval 24–27 min; all them in the negative ionization mode by formation of the formic acid adduct $[M+FA-H]^-$. Two precursor ions were detected within this interval: *m/z* 381.2277 and *m/z* 383.2435 that provided two and

four chromatographic peaks, respectively (see Supplementary Fig. 4). The first was identified as the isomers sterebin I and J and the second as the four isomers sterebin E, F, M and N. These compounds were not fragmented in the collision cell since no fragments were detected in the MS/MS spectra. Thus, their identification was supported on comparison of the monoisotopic mass in the databases for each compound, providing an accuracy error below 2 ppm. These compounds had been previously reported in the literature by Oshima, Saito, & Hikino (1988), McGarvey et al. (2003) and Ibrahim et al. (2007), who isolated and characterized sterebin isomers extracted from Stevia plants.

3.4. Amino acids and derivatives

Amino acids are indispensable for the organism as monomers to form proteins. In humans some of these compounds (essential amino acids) have to be ingested in the diet because the human body cannot synthesize them. Despite their paramount importance in the organism, only two studies have been devoted to the amino acids content of Stevia leaves. First, Esmat Abou-Arab, Azza Abou-Arab, & Ferial Abu-Salem (2010) hydrolyzed proteins and quantified 17 amino acids, among which 8 were essential. More recently Periche et al. (2014) studied the concentration of free amino acids in Stevia leaf infusions using a GC−MS-based method for analysis after derivatization. In this case, 11 amino acids were quantified in the infusions, 7 of them being essential. In the research proposed here, free amino acids in the extracts were studied, resulting in the tentative identification of 13 amino acids and derivatives, among which 5 were essential (they are marked with an asterisk in Table 1); all them in the chromatographic interval 2–3 min, excluding tryptophan, detected at 9.70 min. Identification of the amino acids was carried out searching the immonium ion generated in the fragmentation of the amino acid, as documented by Ledesma-Escobar, Priego-Capote, & Luque de Castro (2015). All amino acids were tentatively identified in the polar extract and detected in the positive ionization mode as $[M+H]^+$ ion or the

$[M+NH_4]^+$ adduct. Proline, choline and serine provided chromatographic peaks among the five most intense detected in this ionization mode; so, they were the most concentrated amino acids and derivatives present in Stevia leaves. Proline, one of the non-essential amino acids that can be classified as essential under given conditions (Otten, Hellwig, & Meyers, 2006), was identified in the extract. Choline, an amino acid derivative, is essential in human nutrition as a part of glycerophosphatidylcholines present in cellular membranes. Finally, serine is a non-essential amino acid for human nutrition with a key role in plants.

3.5. Other families tentatively identified in Stevia extracts

Apart from the families previously commented six additional families were detected in Stevia leaves. First, fatty acid amides and derivatives by conjugation of fatty acids and amino acids were tentatively identified in polar and non-polar extracts in the positive ionization mode, generating the $[M+H]^+$ ion and $[M+Na]^+$ adduct. These compounds have been poorly studied; therefore, most of them were not recorded in the used databases. Identification was carried out by taking as reference the isotopic mass of the equivalent fatty acid, subtracting the –OH group and adding the $–NH_2$ group. Five fatty acid amides were tentatively identified in Stevia leaves, where docosenamide and N-steraroyl valine provided the two most intense peaks. These compounds had previously been detected in plants, as is the case with docosenamide (also named as erucamide), identified in extracts from pitcher plants of the genus *Heliamphora* by Jaffé, Blum, Fales, Mason, & Cabrera (1995).

Two related families, fatty acids and glycerolipids, were also detected in Stevia leaves. These compounds are in all organisms since fatty acids constitute the non-polar part of the lipids and glycerolipids responsible for energy storage. Fatty acids were detected in the negative ionization mode generating the $[M-H]^-$ ion. Glycerolipids were also detected in the positive ionization mode by forming the $[M+Na]^+$ and $[M+NH_4]^+$ adducts. These compounds were tentatively

identified on the basis of their monoisotopic mass since they are very sensitive to fragmentation and they do not produce clear fragments that could allow identification by MS/MS. Thus, a total number of 16 glycerolipids (7 mono-, 8 di- and 1 triglyceride) were tentatively identified in the non-polar extract, while 4 fatty acids and 3 derivatives were identified in both polar and non-polar extracts.

One other common family identified in Stevia leaves was that of oligosaccharides. Four compounds were tentatively identified within this group in both ionization modes generating the $[M-H]^-$ or the $[M+H]^+$ adduct. A particular case is gamma-cyclodextrin, a ring formed by 8 glucoses that generated the $[M+3H]^{3+}$. This compound is considered as soluble fiber and to which fat blocker and anti-obesity properties have been attributed (Artiss, Brogan, Brucal, Moghaddam, & Catherine Jen, 2006; Grunberger, Catherine Jen, & Artiss, 2007).

Finally, two compounds pertaining to different families were identified in the polar extracts of Stevia leaves: purine and a retinol derivative. Both were detected as the $[M+H]^+$ adduct in the positive ionization mode. The peak corresponding to purine was the fourth most intense in the chromatogram. A similar behavior was noted by Frischknecht & Baijmann (1985), who observed a high production of purine alkaloids in the plant *Coffea Arabica* when subjected to stress. A retinol derivative was identified through identification of retinol as precursor ion, but the latter is not present in the plants since it is formed by digestion of precursors such as β-carotene. Thus, the detected compound was identified as a retinol derivative that losses its additional moiety by in source fragmentation, and liberates retinol as precursor ion.

4. Conclusions

Comprehensive profiling of the compounds present in Stevia leaves was achieved using an LC−QTOF MS/MS-based method. A total of 89 compounds

were tentatively identified in the polar and non-polar extracts of the plant, and classified into different families. Steviol glycosides, and quinic and caffeic acids and derivatives resulted to be the two families of compounds that characterized the polar extracts of Stevia. Several steviol glycosides are well-known and have been widely studied, but new steviol glycosides are reported in this study and their structures proposed for the first time. On the other hand, caffeoylquinic acids, here identified and existing in Stevia leaves at high concentrations, have been poorly studied, despite their beneficial properties. Furthermore, free amino acids were identified in the polar extracts from Stevia leaves. Compounds within a poorly-known family (fatty acid amides and derivatives) were tentatively identified after proposing a strategy for their identification. Finally, fatty acids and derivatives and glycerolipids, common compounds in all the organisms, and purine and the retinol derivative were also identified in the extracts. This study reveals the wide range of interesting compounds that are present in Stevia and that endow the extracts with beneficial properties, in addition to the sweetening power of steviol glycosides. These results can lead to further studies of the families here identified to provide benefits to commercial products from Stevia.

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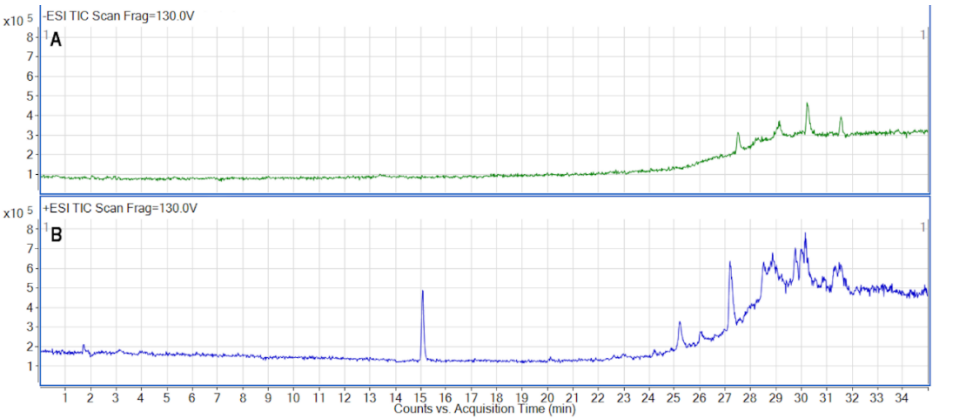
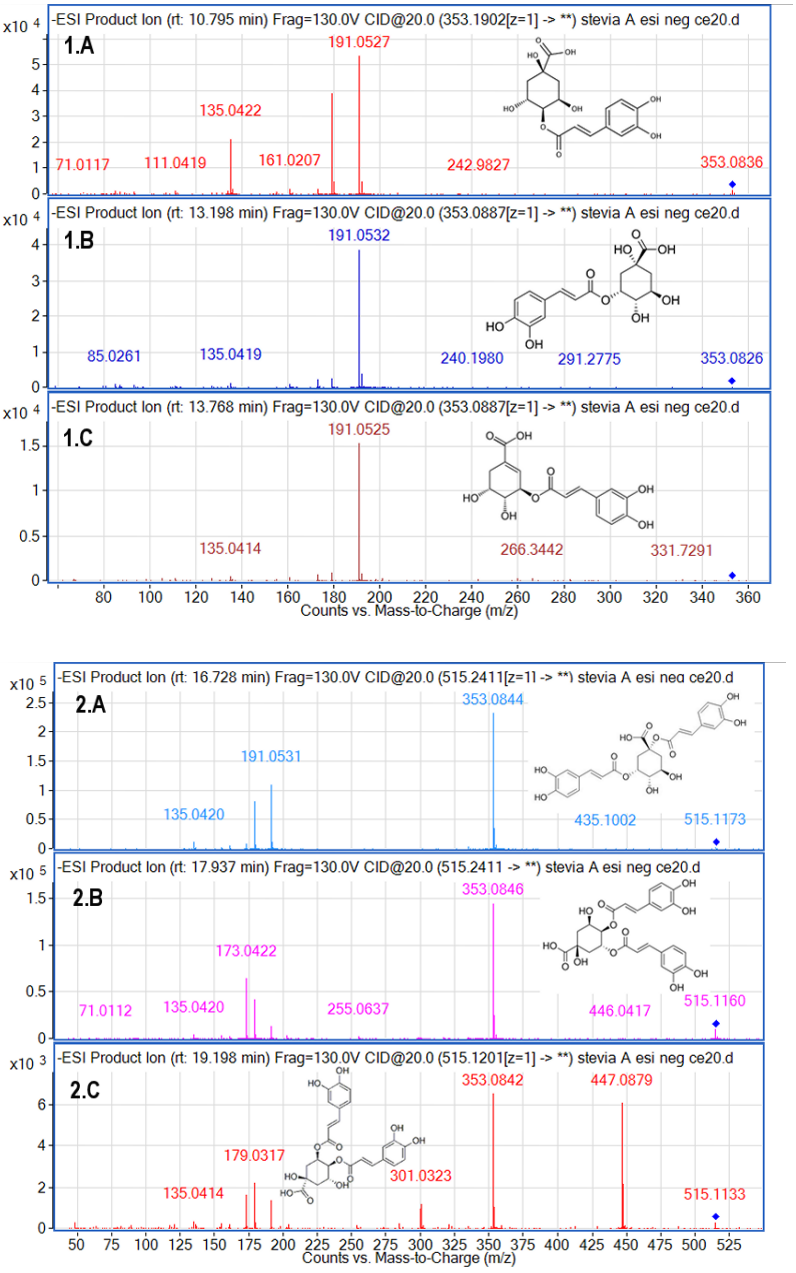
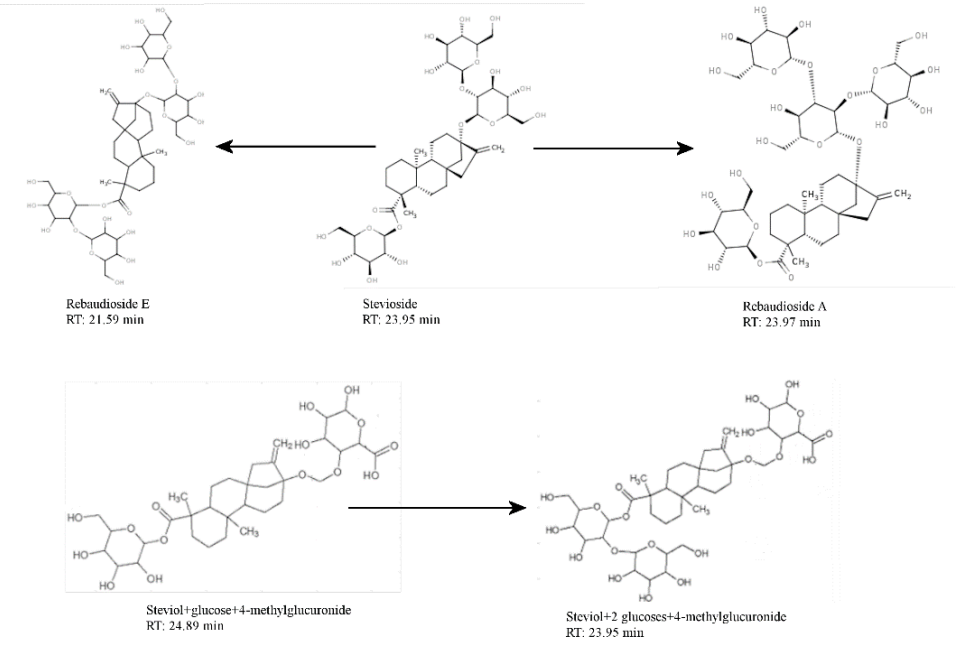
Supplementary information



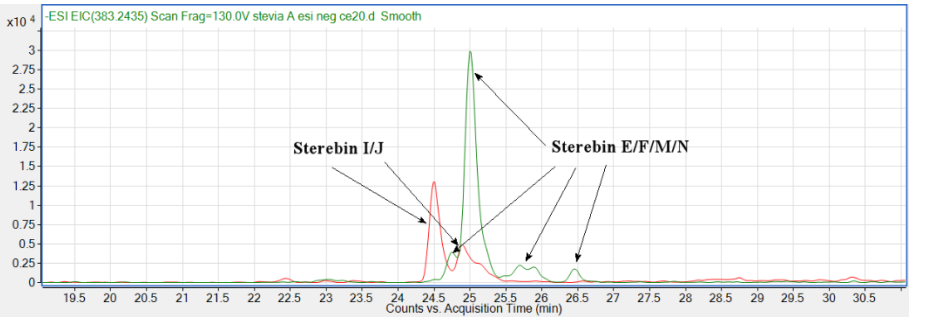
Supplementary Figure 1. MS total ion chromatogram of the non-polar extract in negative (A) and positive (B) ionization modes.



Supplementary Figure 2. MS/MS spectra assigned to 4-caffeoylquinic (1.A), 5-caffeoylquinic (1.B), 3-caffeoylquinic (1.C), 1,5-dicaffeoylquinic (2.A), 4,5-dicaffeoylquinic (2.B) and 3,4-dicaffeoylquinic acid (2.C).



Supplementary Figure 3. Structure of three well-known steviol glycosides (rebaudioside E, stevioside and rebaudioside A) and the proposed structures for two new tentatively identified steviol glycosides (steviol + glucose + 4-methylglucuronide and steviol + 2 glucose + 4-methylglucuronide) and their retention times (RT).



Supplementary Figure 4. Extracted ion chromatogram of the *m/z* 381.2277 corresponding to isomers sterbin I and J, and *m/z* 383.2435 corresponding to the four isomers sterebin E, F, M, and N.

CAPÍTULO V

Development and application of a quantitative method based on LC–QqQ MS/MS for determination of steviol glycosides in Stevia leaves

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Development and application of a quantitative method based on LC−QqQ MS/MS for determination of steviol glycosides in Stevia leaves

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Development and application of a quantitative method based on LC−QqQ MS/MS for determination of steviol glycosides in Stevia leaves

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ABSTRACT

Stevia is a currently well-known plant thanks to the presence of steviol glycosides, which are considered as sweeteners obtained from a natural source. In this research, a method based on LC–MS/MS by using a triple quadrupole detector was developed for quantitation of 8 steviol glycosides in extracts from Stevia leaves. The ionization and fragmentation parameters for selected reaction monitoring were optimized. Detection and quantitation limits ranging from 0.1 to 0.5 ng/mL and from 0.5 to 1 ng/mL, respectively, were achieved: the lowest attained so far. The steviol glycosides were quantified in extracts from leaves of seven varieties of Stevia cultivated in laboratory, greenhouse and field. Plants cultivated in field presented higher concentration of steviol glycosides than those cultivated in greenhouse. Thus, the way of cultivation clearly influences the concentration of these compounds. The inclusion of branches together with leaves as raw material was also evaluated, showing that this inclusion modifies, either positively or negatively, the concentration of steviol glycosides.

Keywords Stevia leaves; steviol glycosides; sweetening; quantitation; triple quadrupole; branches.

1. Introduction

Stevia is a shrub of the Asteraceae family native to South America, especially to Northeast of Paraguay [1]. Presently, the well-known sweet taste of Stevia leaves has made this plant a powerful alternative to artificial sweeteners and has spread Stevia crops to other regions of the world such as Canada, Asia or Europe [2]. Compounds responsible for sweet taste of Stevia have been identified as conjugated glycosides of the diterpene steviol. Stevioside was the first steviol glycoside purified in 1931. However, its structure was not elucidated until 1952. Other compounds, including rebaudioside A with a sweetener power even higher than stevioside, were isolated during the 1970s [3]. Studies on evaluation of the individual sweetener power of these compounds established that the quality differs depending on their structure and the number of glucose molecules conjugated with steviol [4]. Additionally, it should be pointed out that stevioside produces a significant bitter aftertaste [5]; therefore, there is a growing interest in the selective extraction of rebaudiosides over stevioside to increase the sweetener power and prevent the bitter taste. Rebaudioside A and stevioside have been approved as food additives in many countries over the world. The European Food Safety Authority (EFSA) issued a positive scientific opinion on their safety and established the acceptable daily intake for steviol glycosides [6]. In addition to their sweetening properties, some studies suggest that Stevia extracts have therapeutic properties as they possess recognized anti-hyperglycemic, anti-hypertensive, anti-inflammatory, anti-tumor, anti-diarrhea, diuretic and immunomodulatory effects [7]. Furthermore, toxicological studies have shown that steviosides do not have mutagenic, teratogenic or carcinogenic effects, in contrast to artificial sweeteners [8].

Many researchers have evaluated different methods for extracting steviol glycosides using different techniques, from conventional alternatives — maceration [9], extraction with hot water [10,11], or Soxhlet extraction [12]— to

complex extraction techniques based on supercritical CO_2 [13] or on ultrasound-assistance [14,15]. A large variety of solvents has been used for the extraction of steviol glycosides with methanol, ethanol, water [16,17], and also isopropanol [18]. Among the existing methods, the optimum extraction time ranges from 30 min [19] to 48 h [20]. Also different pHs have been reported for the extraction of steviol glycosides, from acid [10] through neutral [21] to alkaline [22]. Despite attempts to propose a definitive protocol for extraction of these compounds, the differentiating optimum working conditions have so far hindered to reach consensus.

Liquid chromatography (LC) is the most widely used technique for separation of steviol glycosides prior to detection, although some authors have tested the use of capillary electrophoresis (CE) as an alternative. Ayyappa *et al.* for example studied the effect of the main CE parameters on the migration and separation of rebaudioside A and stevioside [23]. In studies based on LC, most authors have used HPLC or UPLC equipment, while Morlock *et al.* evaluated the use of high performance thin layer chromatography (HPTLC) for the separation of steviol glycosides and compared this technique with HPLC [24]. Despite the method based on HPTLC resulted to be faster and cheaper, it could not achieve the same resolution as HPLC. In HPLC methods, amino-based columns used in the earliest studies provided a high selectivity for all steviol glycosides and enough resolution for the most abundant isomer pairs. However, the disadvantages of this sorbent are poor reproducibility and long equilibration times that make it not suitable for individual separation of steviol glycosides [25]. Therefore, C18 phase columns have also been used for separation of steviol glycosides including the aglycone form. These columns are robust, but their poor selectivity does not allow complete separation of isomers [19]. Combined amino and C18 columns provide a better separation of steviol glycosides [26], necessary when UV detectors at absorption wavelengths between 200 and 300 nm are used [27,28]. Because of the relatively

low acquisition and maintenance costs of these detectors, they have been widely used after LC separation, despite not being sensitive enough for the analysis of some steviol glycosides. This weakness is widely surpassed by using tandem mass spectrometry (MS/MS) detectors that have a higher sensitivity and the capability for discrimination between pairs of isomers by applying the selected reaction monitoring (SRM) mode. Some studies dealing with MS/MS for quantitative analysis of steviol glycosides have been published. Shafii *et al.* and Well *et al.* used a quadrupole-ion trap detector for the quantitation of 9 and 10 steviol glycosides, respectively, in Stevia plants [17,29]; Shah *et al.* evaluated the concentration of steviol glycosides in Stevia podwer, drinks and liquid sweetener by LC–MS/MS with a triple quadrupole detector [30]. Also, Gardana *et al.* and Periche *et al.* used a triple quadrupole detector for quantitation of 4 and 5 steviol glycosides, respectively, in Stevia leaves [31,32], as also did Montoro *et al.* for the quantitative analysis of six steviol glycosides in extracts from Stevia plants cultivated in different countries [33]. In this last case, electrospray ionization (ESI) in positive ionization mode was used, but no results were provided from the negative ionization mode. One other similar method was proposed by Espinoza *et al.*, in which an LTQ-Orbitrap MS in negative ionization mode was used. In this particular case, nine steviol glycosides were identified in commercial Stevia extracts. Quantitative analysis was carried out by SRM to correlate the sweetener power with the results provided by tasting, but no analytical validation was carried out [34].

The primary aim of the research here presented was to develop a method for quantitative analysis of the main steviol glycosides based on LC–MS/MS in SRM mode to evaluate the sweetening capability of Stevia leaves. The applicability of the method was demonstrated by quantifying these compounds in leaves from Stevia plants cultivated in different media (the laboratory, the greenhouse and on the field). Additionally, the use of branches together with leaves as raw material

for extraction of steviol glycosides was evaluated.

2. Experimental

2.1. Samples, standards and reagents

Leaves from different varieties of *Stevia rebaudiana* cultivated in the South of Spain in laboratory (7 varieties named from LAB1 to LAB7), greenhouse (7 varieties from GH1 to GH7) and field (7 varieties from FIELD1 to FIELD7) were provided by Vitrosur Lab. S.L.U. (Los Palacios y Villafranca, Sevilla, Spain). Samples were collected from the plant 60 days after transplanting. Additionally, samples containing leaves plus branches were sampled from the varieties cultivated in field to evaluate the influence of the sampling process on the content of steviol glycosides. Stevioside, rebaudioside A, B, C and D, steviolbioside, rubusoside and dulcoside A were from Extrasynthese (Genay, France). All reagents were of analytical grade or higher. Deionized water (18 MΩ·cm) was obtained by a Milli-Q water purification system from a Millipore (Bedfore, MA, USA) Milli-Q plus system, LC-grade methanol was from Panreac (Barcelona, Spain) and LC-grade ethanol from Scharlab (Barcelona, Spain).

2.2. Extraction step

Steviol glycosides were extracted from either Stevia leaves or leaves+branches by maceration for 2 h using 25 mL of an ethanol–water (35:65 v/v) mixture as extractant. To confirm quantitative extraction, a re-extraction of the solid residue was carried out resulting in a concentration lower than 1% of that in the first extract.

2.3. Apparatus and instruments

An Agilent 1200 Series LC system from Agilent Technologies (Palo Alto, USA) with a 6460 Triple Quad LC–MS detector equipped with a Jet Stream Technology electrospray ion source, also from Agilent, was used to carry out analyses of the extracts. A Mediterranea Sea C18 analytical column (5 µm, 15 × 0.46

cm) from Teknokroma (Barcelona, Spain) was used for the separation together with deionized water with 0.1% of formic acid (A) and methanol with 0.1% of formic acid (B) as mobile phases. The gradient was as follows: start with 60% B, change from 60% to 100% B in 15 min and constant 100% B for 5 min more. A post-run of 5 min was programmed to equilibrate the column between analyses. The flow rate was constant at 0.7 mL/min and the injected volume 10 μL. The column temperature was set at 40 ºC.

High-purity nitrogen (99.999%) as collision gas was used in the triple quadrupole MS. Determination of the compounds was performed by ESI-MS/MS in SRM mode. The sheath gas flow and temperature were set at 12 mL/min and 350 ºC, respectively, and nebulizer gas temperature was set at 325 ºC. The pressure of the nebulizer was 45 psi, the capillary voltage was set at 6000 V and the nozzle voltage at 1000 V for the negative ionization mode. Dwell time was set at 50 ms for each SRM transition.

2.4. Quantitation of steviol glycosides

The calibration curves were built by plotting the peak area of each steviol glycoside versus its nominal concentrations present in multistandard solutions. For this purpose, 12 calibration levels were prepared in methanol with the target analytes at suited concentrations to define the calibration ranges and evaluate the sensitivity of the quantitation method by estimation of the limits of detection and quantitation (LODs and LOQs, respectively).

3. Results and discussion

3.1. Optimization of the SRM detection mode

As previously stated, LC–QqQ MS/MS studies for quantitative analysis of steviol glycosides used either positive or negative ionization mode but few of them carried out a comparison between the ionization modes. In the present study both

ionization modes were compared. Concerning separation, methods that used reverse phase chromatography showed high resolution, except for some pairs of isomers. Nevertheless, as a QqQ detector was selected for this research, chromatographic resolution was not a limiting factor and a C18 column was used for development of the method.

The main parameters involved in MS ionization and fragmentation were optimized to achieve the highest sensitivity in detection. The ionization operating conditions were studied by direct injection of individual standard solutions of each analyte at 5 μg/mL using the positive and negative ionization modes. The negative ionization mode showed a more efficient ionization by generation of $[M–H]^-$ and $[M–Glu]^-$ precursors providing the best sensitivity for the target compounds as compared to the precursor ions in positive mode, $[M+H]^+$. This result is in agreement with the study of Shah *et al.*, who also found negative ionization the best option for quantitation of steviol glycosides [30]. The precursor ions of stevioside, rebaudioside A and dulcoside A underwent a loss of a glycoside moiety by in-source fragmentation in the electrospray unit to generate the $[M–Glu]^-$ ion, as can be seen in the scan spectra in Suppplementary Fig. 1. The $[M–H]^-$ ions presented a very low intensity as compared to those formed by loss of a glucose moiety and, in the case of stevioside and dulcoside A, the $[M+Cl]^-$ adducts were also observed, but they showed a low reproducibility. For the remaining steviol glycosides, the negative ionization mode favored the formation of $[M–H]^-$ as precursor ions. After selection of the precursor ions, the parameters of the ESI source were optimized, as shown in Table 1. Particularly, the ionization of the target analytes made necessary nebulizer gas temperature (N_2 for nebulization of the eluate from the chromatograph) and sheath gas temperature (N_2 for focusing the nebulized flow) above 300 ^oC, which are characteristic of non-thermolabile compounds. The capillary voltage was set at 6 kV to force the ionization process, while the nozzle voltage was set at an intermediate value (1000 V).

Detection of each compound was based on the SRM mode. Thus, MS/MS parameters were optimized for efficient isolation of the precursor ions and their sensitive and selective fragmentations. Voltage of the first quadrupole for an efficient filtration of the precursor ions, collision energy and selected product ions for the quantitation transitions were optimized. As Fig. 1 and 2 show, steviol glycosides, excluding dulcoside A, showed a characteristic transition generated by the loss of *m/z* 162.1 from the precursor ion, which corresponds to a glycoside residue. Concerning dulcoside A, its SRM transition was produced by the loss of *m/z* 146.1 forming the product ion, identified as the ion resulting from cleavage of the rhamnose residue.

The SRM method was characterized by a high selectivity thanks to chromatographic and/or MS resolution. It is worth mentioning that rebaudioside A and rebaudioside B were quantified using the same SRM transition. However, this was not a problem since both steviol glycosides were chromatographically separated eluting at 8.3 and 10 min, respectively. Similar behavior was observed for steviolbioside and rubusoside. On the other hand, the pairs stevioside/rebaudioside A, reabudioside C/dulcoside A and rebaudioside D/rubusoside coeluted with the chromatographic gradient used, but their SRM transitions in the MS/MS mode were different and allowed their selective quantitation.

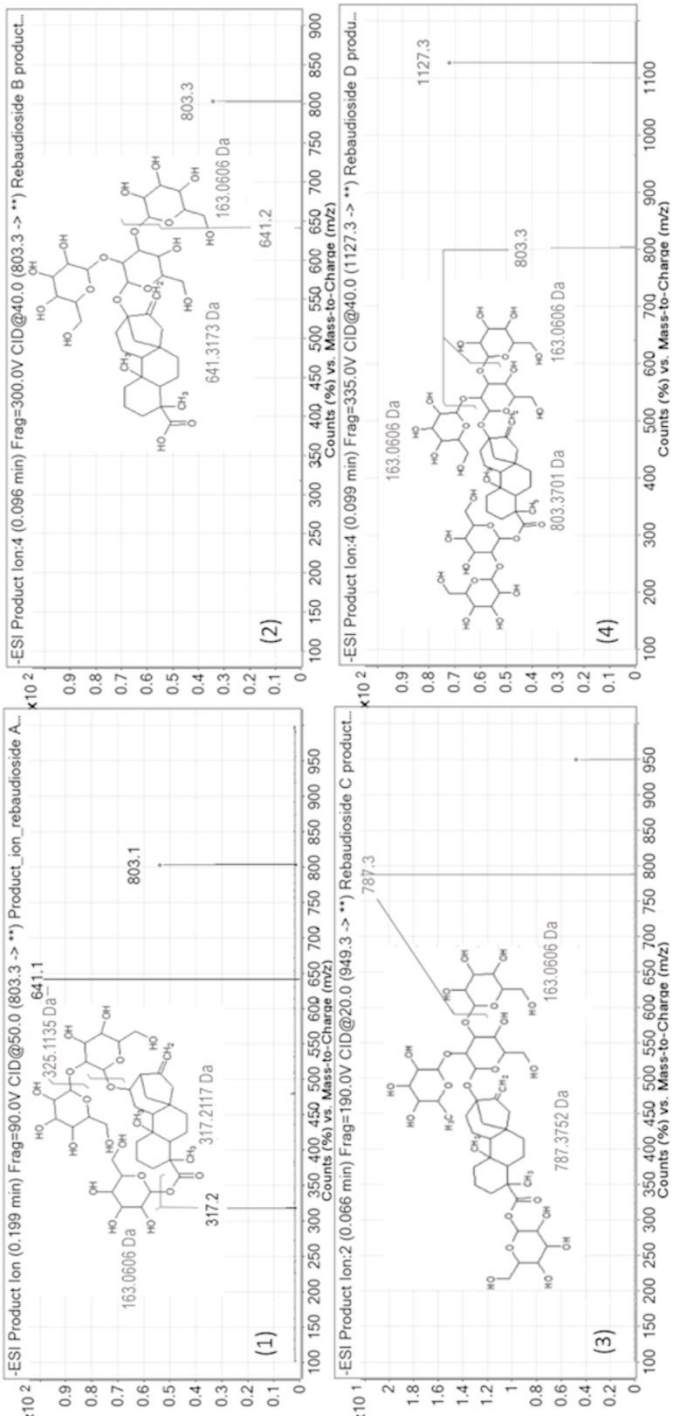

Figure 1. MS/MS spectra and fragmentation patterns of rebaudioside A (1), B (2), C (3) and D (4).

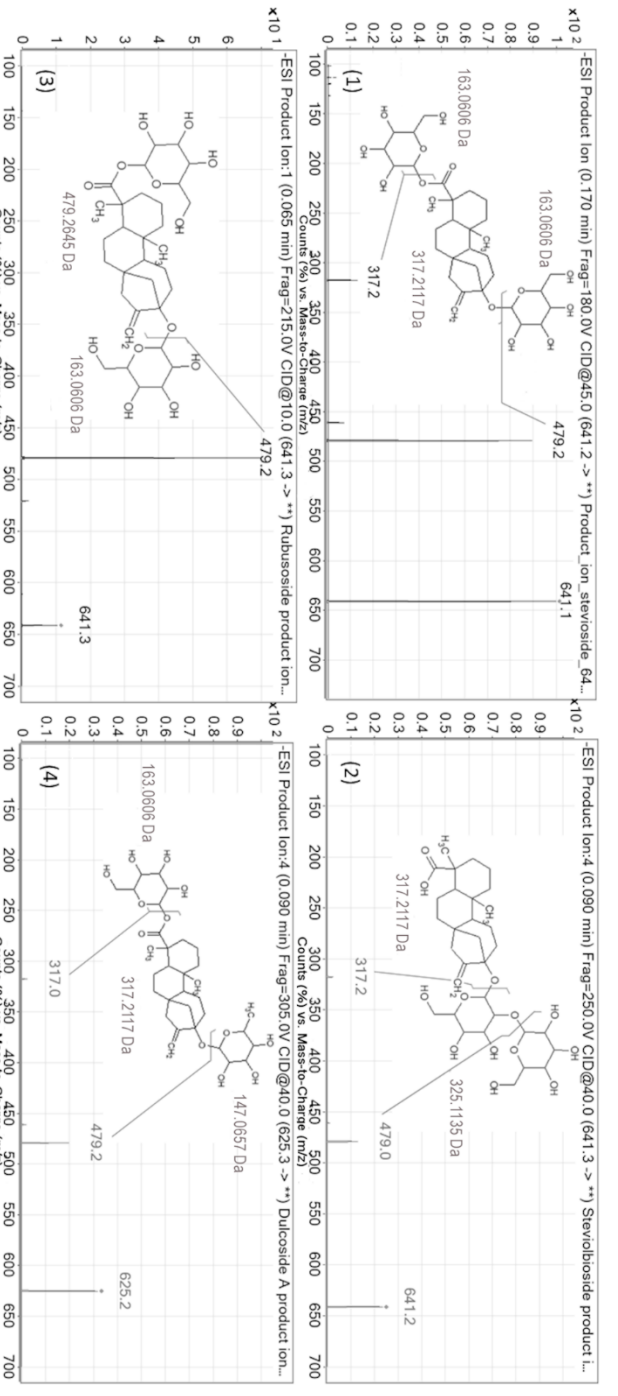

Figure 2. MS/MS spectra and fragmentation patterns of steviolbioside (1), dulcoside A (2), stevioside (3) and rubusoside (4).

Table 1. Parameters of the LC–MS/MS method for qualitative and quantitative analysis of steviol glycosides.

Compound	Retention time (min)	Mass (Da)	Transition	Quadrupole voltage (V)	Collision energy (eV)
Rebaudioside D	6.1	1129.15	1127.3→803.2	335	40
Rebaudioside A	8.3	967.01	803.1→641.1	90	50
Stevioside	8.3	804.97	641.3→479.2	180	45
Rebaudioside C	8.8	951.01	949.3→787.2	190	20
Dulcoside A	8.8	788.87	625.3→479.2	305	40
Steviolbioside	9.4	642.73	641.3→479.2	250	40
Rubusoside	10.0	642.73	641.3→479.2	215	10
Rebaudioside B	10.0	804.87	803.3→641.2	300	40

Variable	Tested range	Optimal value
Gas temperature (°C)	0–350	325
Sheath gas temp (°C)	0–400	350
Capillary voltage (V)	0–6000	6000
Nozzle voltage (V)	0–2000	1000

Table 2. Analytical features of the method for determination of steviol glycosides.

Compound	Calibration equation (0-0.5 µg/mL)	Regression coefficient	Calibration equation (0.5-5.0 µg/mL)	Regression coefficient	LOD (ng/mL)	LOQ (ng/mL)
Rebaudioside D	y=53232x + 28.812	0.9985	y=58589x – 790.71	0.9976	0.1	0.5
Rebaudioside A	y=142790x + 100.02	0.9982	y=83308x + 14843	0.9885	0.1	0.5
Stevioside	y=128202x + 104.55	0.9969	y=68880x + 15771	0.9797	0.1	0.5
Rebaudioside C	y=440922x – 358.13	0.9969	y=230345x + 40957	0.9944	0.1	0.5
Dulcoside A	y=233033x +182.72	0.9973	y=110633x + 27824	0.9823	0.5	1
Steviolbioside	y=89985x +49.392	0.9981	y=51729x + 9974.3	0.9892	0.1	0.5
Rubusoside	y=587150x + 405.22	0.9974	y=248248x + 64861	0.9893	0.1	0.5
Rebaudioside B	y=41835x + 24.614	0.9974	y=25168x + 4435	0.9903	0.5	1

3.2. Linearity of calibration curves and lower limits of quantitation

A linear regression model was applied to generate two calibration curves in two different concentration ranges for each analyte: from LOQ values to 0.5 μg/mL and from 0.5 to 5.0 μg/mL. The regression coefficients (R^2) of the calibration models built for the lowest concentration range were higher than 0.99 (see Table 2), indicative of an excellent fitting. On the other hand, the calibration models defined for the highest concentration range were characterized by R^2 values above 0.98.

The sensitivity of the method was evaluated by estimation of the limits of detection (LODs) and quantitation (LOQs) for each analyte by injecting dilution series of a multistandard solution to obtain the concentration which provided signals three and ten times the background noise, respectively (Table 2). The LOQs for glycosylated steviol forms were within the range 0.5–1.0 ng/mL, while the LODs were within 0.1–0.5 ng/mL.

Comparison of the sensitivity of the proposed method with the values given in literature is shown in Supplementary Table 1. Wang *et al.* (2015) provided the highest LOD and LOQ values using a UV detector, which is much less sensitive than the MS detectors used in the other listed studies. High values of these parameters were obtained in the study of Aranda-González *et al.* (2015), who reported LOD and LOQ in the range of μg/mL also using a UV detector. On the other hand, Periche *et al.* (2015) proposed a method using LC–MS/MS with triple quadrupole, but the quantitation was carried out in the positive ionization mode. Although the LOD and LOQ values were lower than those given by Wang *et al.* and Aranda-Gonzalez *et al.*, they did not reach the sensitivity achieved in the negative ionization mode. This ionization mode was used by Montoro *et al.* (2013), who reached LOQs and LODs in the ranges of 2.1–7.8 ng/mL and 0.1–2.8 ng/mL, respectively, using LC–MS/MS with triple quadrupole for quantitation of six steviol glycosides. Also Shafii *et al.* (2012) proposed a quantitation method using

similar equipment and achieving LODs within the 0.7–14.7 ng/mL range. Comparing the LODs and LOQs previously reported with those obtained in this study, it is worth pointing out that the method here proposed provided a higher sensitivity thanks to the optimization of all the parameters involved in both steps, ionization and fragmentation.

3.3. Application of the method to Stevia leaf extracts

The proposed LC–MS/MS method was applied to the analysis of the target compounds in different varieties of Stevia cultivated under different conditions, described in the experimental part (2.1.). Supplementary Table 2 lists the concentration of steviol glycosides found in the analyzed samples in absolute terms expressed as μg/g of dry leaves. Also shown are the relative concentrations of each compound as percentage of the total steviol glycoside content. Stevioside and rebaudioside A were the two major glycoside forms of steviol in all samples, with concentrations ranging from 16 to 55% and from 36 to 55%, respectively. The concentration of each steviol glycoside in the target samples is shown as bar diagram in Fig. 3, with the compounds grouped by their concentration levels. In overall terms, the Stevia varieties cultivated in the laboratory contained concentrations of steviol glycosides much lower than the rest of the samples, in some cases 100 times lower. The samples with the highest concentrations of steviol glycosides were the varieties cultivated in the field, especially rich in stevioside and rebaudioside A, B, C and D. Among the varieties cultivated in the field, sample FIELD3 is noteworthy for its high content in stevioside, rebaudioside A, rubusoside and dulcoside A, although the content of the rest of rebaudiosides was particularly low. By comparing the obtained results with those provided by Mantovaneli *et al.* (2004) concerning the sweetening power of these compounds relative to the sweetening power of sucrose as the reference (see Supplementary Table 3), rebaudioside A, B and D are the steviol glycosides with the highest sweetening power. Thus, the most interesting varieties in this context would be

those richer in these compounds. With this criterium, sample FIELD2 seems to be the most interesting thanks to the high content in rebaudioside A and D, since its extracts will provide the highest sweetening capacity.

Figure 3. Concentration of steviol glycosides (µg/g) in leaves from Stevia varieties cultivated in laboratory (LAB), greenhouse (GH) and field (FIELD).

3.4. Comparison of Stevia leaves collected with and without branches

The samples of the varieties cultivated in the field were collected in two ways: selecting only the leaves and collecting the branches together with the leaves. The comparison of the results of each type of sample aimed at detecting the contribution of branches to the concentration of steviol glycosides. Supplementary Table 4 shows the concentrations of steviol glycosides in leaves (L) and

leaves+branches (L+B). The values obtained in each case are plotted in Fig. 4, showing that in the extracts from samples containing branches the concentration of steviol glycosides is lower than in those from samples with only leaves, especially for samples F4 and F7, where the content of steviol glycosides is drastically reduced (more than 50%) when branches are included in the sample. Sample F5 must be excluded from this behavior since it provided an extract with slightly higher concentration of steviol glycosides in L+B than in L; therefore, the branches of this variety contain a higher amount of steviol glycosides than leaves.

The results were statistically treated by a paired *t*-test to detect if the steviol glycoside content was significantly different in samples with and without branches (p-value < 0.05). The *t*-test allowed detecting significant differences in the concentration of stevioside (p-value 0.0163), rebaudioside A (p-value 0.019), rebaudioside C (p-value 0.0090), steviolbioside (p-value 0.0132), rubusoside (p-value 0.0446) and dulcoside A (p-value 0.0295). These values show that only the levels of rebaudioside B and D were not significantly different in the two types of samples, while rebaudioside C was the compound most affected by the inclusion of branches in the raw material. The paired *t*-test was also applied to the overall concentration of all steviol glycosides (total content) and to the sum of the compounds with the highest sweetening power (see Supplementary Table 3) as exposed above, which included stevioside, rebaudioside A, B and D, as well as the sum of the compounds with the lowest sweetener power, including rebaudioside C, steviolbioside, rubusoside and dulcoside A. These three parameters were also found significant to assess how the concentrations of steviol glycosides are modified when branches are included in the raw sample (p-value 0.0142, 0.0148 and 0.0122, respectively). These results can be visualized in the Box-and-Whisker corresponding to compounds or parameters for which the type of sample was statistically significant (see Supplementary Fig. 2).

Therefore, the collection of branches with leaves showed that the content of

steviol glycosides is significantly different from that of leaves (in most cases this content decreased). This difference is dependent on the Stevia variety since the compounds are not all affected in the same way. Rebaudioside B and D, for example, do not change significantly in their concentration when branches are included for extraction.

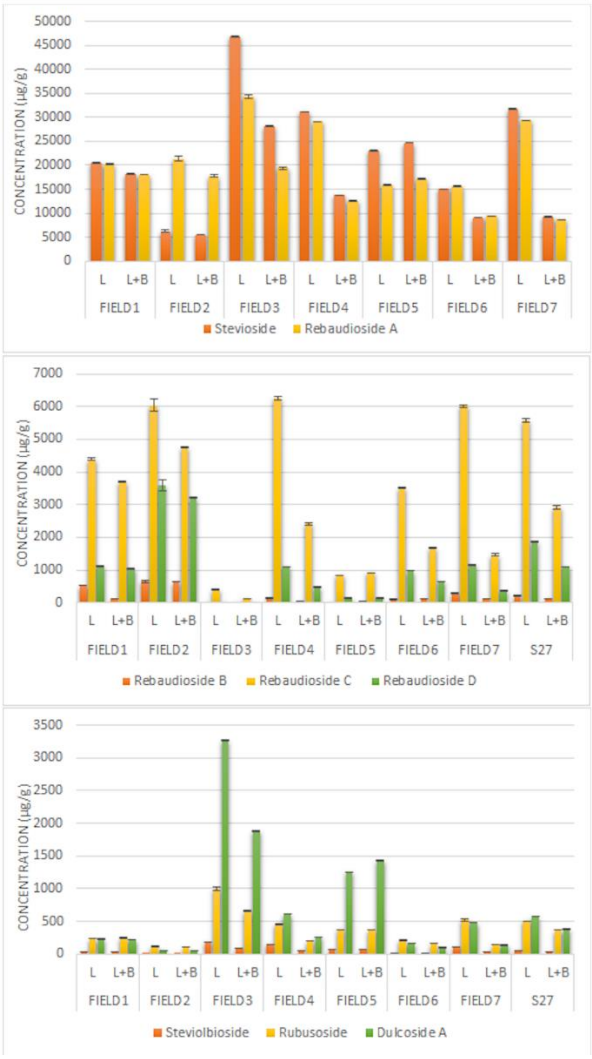

Figure 4. Concentration of steviol glycosides (µg/g) in Stevia varieties cultivated in field by sampling leaves (L) and leaves+branches (L+B).

4. Conclusions

An LC–ESI–MS/MS method is proposed for the quantitation of steviol and its glycosylated forms. This method allows to discern between steviol glycosides that show isomerism, and provided LODs and LOQs lower than those obtained in other research, thus improving the sensitivity for the analysis of these compounds. The analysis of Stevia varieties by this method proved that the content in steviol glycosides is drastically dependent on the type of cultivation. Thus, this content was reduced when cultivated in greenhouse as compared to in field. Finally, the evaluation of the incorporation of branches to the samples showed that the content of steviol glycosides is affected by the presence of branches, but the effect (in most cases a reduction in steviol glycosides content) is dependent on the variety of Stevia. Also, not all compounds are affected in the same way since only rebaudioside B and D do not present significant differences in concentration when branches are included in the sample.

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Conflict of interest

The Stevia varieties resulted from given crosses and were provided by Vitrosur Lab S.L.U. after signing a non-disclosure agreement (NDA).

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Supplementary information

Supplementary Table 1. Comparison of LOD and LOQ values provided by the proposed method with other studies found in the literature.

	Quantified compounds	LOD (ng/mL)	LOQ (ng/mL)
Wang *et al.*	5	10000	30000
Aranda-González *et al.*	4	5000–8000	17000–26000
Periche *et al.*	5	40–140	150–490
Montoro *et al.*	6	0.1–2.8	2.1–7.8
Well *et al.*	10	–	1.16–27.36
Shafii *et al.*	9	0.7–14.7	2.3–47.6
Gardana *et al.*	4	1–50	5–100
Shah *et al.*	10	0.05–3.35	–
Kubica *et al.*	6	1.08–4.52	3.23–13.56
Proposed method	8	0.1–0.5	0.5–1.0

Supplementary Table 2. Concentration of steviol glycosides and percentage of the total content of these compounds in leaves from Stevia varieties cultivated in laboratory (LAB), greenhouse (GH) and field (FIELD).

	Stevioside		Rebaudioside A		Rebaudioside B		Rebaudioside C		Rebaudioside D		Steviolbioside		Rubusoside		Dulcoside A	
	µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%
LAB1	9786 ± 349	41.16	9190 ± 335	38.65	52.3 ± 9.7	0.22	3196 ±173	13.44	459 ± 22	1.93	233 ± 10	0.98	18.6 ± 0.5	0.08	842 ± 14	3.54
LAB2	12691 ± 171	45.32	10811 ± 184	38.61	28.8 ± 4.0	0.10	2159 ± 24	7.71	266 ± 3	0.95	446 ± 31	1.59	59.7 ± 3.7	0.21	1540 ± 7	5.50
LAB3	526 ± 3	51.73	431 ± 2	42.34	1.7 ± 0.1	0.16	37.4 ± 0.1	3.68	7.5 ± 0.3	0.74	3.3 ± 0.1	0.32	1.6 ± 0.0	0.15	9.0 ± 0.1	0.88
LAB4	1598 ± 13	57.78	1085 ± 25	39.22	0.0 ± 0.0	0.00	9.9 ± 0.1	0.36	0.5 ± 0.1	0.02	11.1 ± 0.3	0.40	4.4 ± 0.4	0.16	57.0 ± 1.5	2.06
LAB5	4344 ± 34	51.26	3545 ± 33	41.83	11.5 ± 0.6	0.14	365 ± 3	4.31	88.2 ± 1.7	1.04	39.0 ± 0.8	0.46	11.0 ± 0.4	0.13	70.1 ±0.6	0.83
LAB6	992 ± 5	49.13	743 ± 4	36.81	3.2 ± 0.6	0.16	47.3 ± 0.5	2.34	14.2 ± 1.1	0.70	4.8 ± 0.2	0.24	197.0 ± 2.5	9.76	16.6 ± 0.1	0.82
LAB7	644 ± 5	55.07	462 ± 6	39.55	0.6 ± 0.1	0.05	29.7 ± 0.7	2.54	6.4 ± 0.1	0.55	3.2 ± 0.1	0.27	3.8 ± 0.1	0.32	19.2 ± 0.3	1.64
GH1	9648 ± 97	52.44	7526 ± 124	40.91	0.0 ± 0.0	0.00	790 ±13	4.30	52.4 ± 1.7	0.28	53.2 ± 0.3	0.29	62.9 ± 2.3	0.34	257 ± 8	1.40
GH2	10989 ±193	51.99	8619 ± 296	40.78	12.9 ± 2.0	0.06	936 ± 19	4.43	78.7 ± 3.0	0.37	56.0 ± 0.3	0.26	48.0 ± 0.7	0.23	397 ± 13	1.88
GH3	7135 ± 65	36.52	7987 ± 42	40.88	212.1 ± 7.2	1.09	3572 ± 66	18.29	485 ± 17	2.48	14.6 ± 4.6	0.07	16.8 ± 0.1	0.09	115.0 ± 16.6	0.59
GH4	8138 ± 242	51.91	6382 ± 198	40.71	12.1 ± 1.2	0.08	748 ± 6	4.77	31.5 ± 1.6	0.20	36.5 ± 14.7	0.23	80.8 ± 2.0	0.52	249.4 ± 2.9	1.59
GH5	4666 ± 55	51.81	3509 ± 117	38.96	0.0 ± 0.0	0.00	579 ± 17	6.43	12.7 ± 0.8	0.14	42.2 ± 4.4	0.47	112.8 ± 5.2	1.25	84.9 ± 1.6	0.94

Cont. Supplementary Table 2

GH5	4666 ± 55	51.81	3509 ± 117	38.96	0.0 ± 0.0	0.00	579 ± 17	6.43	12.7 ± 0.8	0.14	42.2 ± 4.4	0.47	112.8 ± 5.2	1.25	84.9 ± 1.6	0.94
GH6	15834 ± 223	49.88	12499 ± 125	39.37	22.9 ± 3.4	0.07	1663 ± 93	5.24	101.8 ± 2.6	0.32	316.2 ± 14.3	1.00	138.4 ± 2.1	0.44	1170 ± 11	3.68
GH7	8136 ± 153	51.72	6441 ± 225	40.95	0.0 ± 0.0	0.00	866 ± 90	5.51	52.7 ± 2.2	0.34	101.3 ± 3.9	0.64	23.1 ± 0.4	0.15	109.0 ±37.1	0.69
FIELD 1	20447 ± 265	43.24	20311 ± 303	42.96	522.8 ± 15.7	1.11	4387 ± 76	9.28	1117 ± 8	2.36	34.3 ± 0.9	0.07	232.3 ± 1.6	0.49	231 ± 2	0.49
FIELD 2	6227 ± 586	16.64	21343 ± 1084	55.40	649.4 ± 80.2	1.74	6042 ± 380	16.15	3592 ± 362	9.60	5.0 ± 1.5	0.01	115.5 ± 10.6	0.31	54.5 ± 3.8	0.15
FIELD 3	46896 ± 231	54.49	34295 ± 852	39.85	0.0 ± 0.0	0.00	401 ± 2	0.47	24.3 ± 2.3	0.03	183.9 ±2.6	0.21	1003 ± 65	1.17	3263 ± 22	3.79
FIELD 4	31196 ± 30	45.20	29114 ± 127	42.18	139.6 ± 7.7	0.20	6250 ± 93	9.05	1100 ± 18	1.59	146.7 ± 11.7	0.21	465 ± 18	0.67	614 ± 10	0.89
FIELD 5	23109 ± 94	55.44	15885 ± 133	38.11	0.0 ± 0.0	0.00	848 ± 6	2.03	142 ± 8	0.34	73.1 ± 1.0	0.18	372 ± 3	0.89	1256 ± 7	3.01
FIELD 6	15023 ± 63	42.20	15608 ± 230	43.85	100.7 ± 9.0	0.28	3499 ± 44	9.83	971 ± 7	2.73	20.6 ± 0.6	0.06	211 ± 11	0.59	163.5 ± 3.9	0.46
FIELD 7	31698 ± 158	45.50	29405 ± 60	42.20	285.5 ± 15.2	0.41	6020 ± 69	8.64	1147 ± 11	1.65	115.7 ±1.1	0.17	523 ± 23	0.75	480 ± 8	0.69

Supplementary Table 3. Sweetening power of steviol glycosides referred to as sucrose (Mantovaneli *et al.*, 2004).

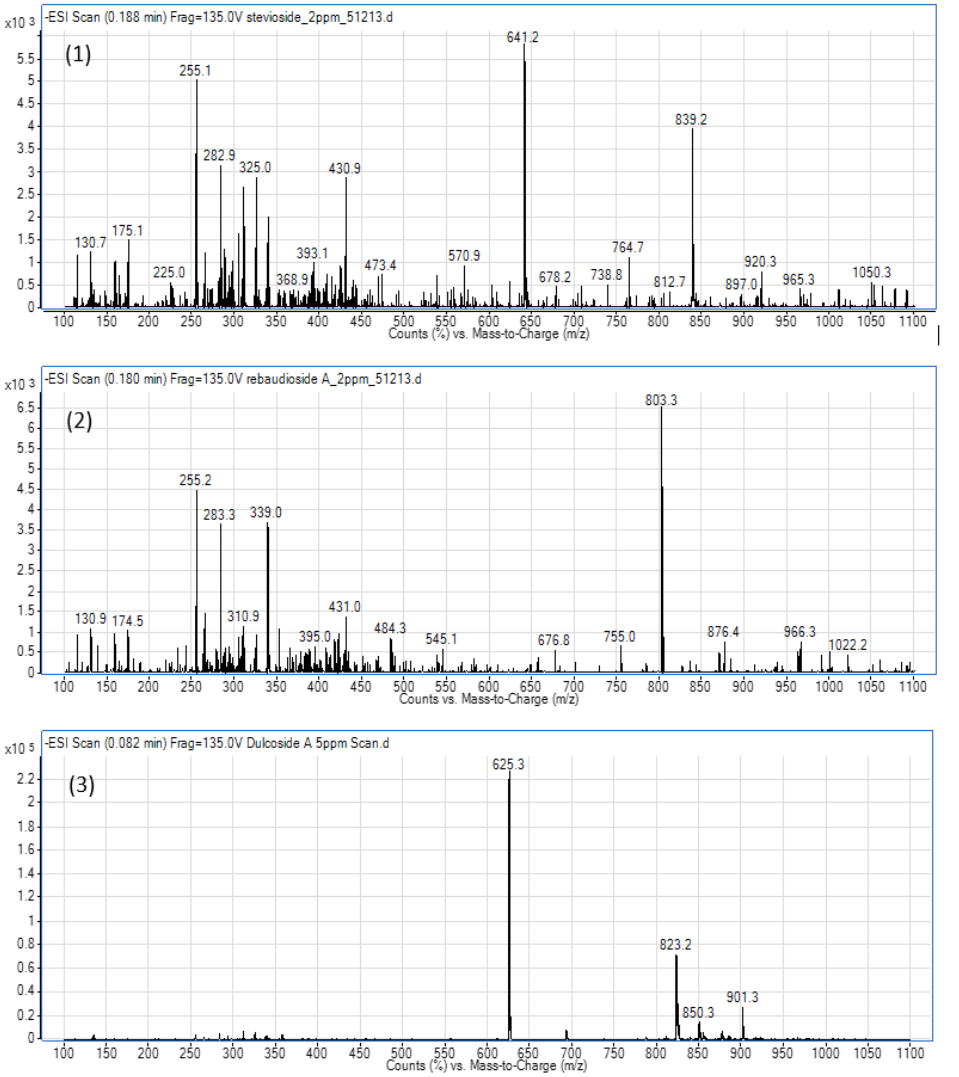
Compound	Sweetening power (Sucrose = 1)
Stevioside	150 – 300
Rebaudioside A	250 – 450
Rebaudioside B	300 – 350
Rebaudioside C	50 – 120
Rebaudioside D	250 – 450
Steviolbioside	100 – 125
Rubusoside	100 – 120
Dulcoside A	50 – 120

Supplementary Table 4. Concentration of steviol glycosides and percentage of the total content of the compounds in Stevia varieties cultivated in field by sampling leaves (L) or leaves+branches (L+B).

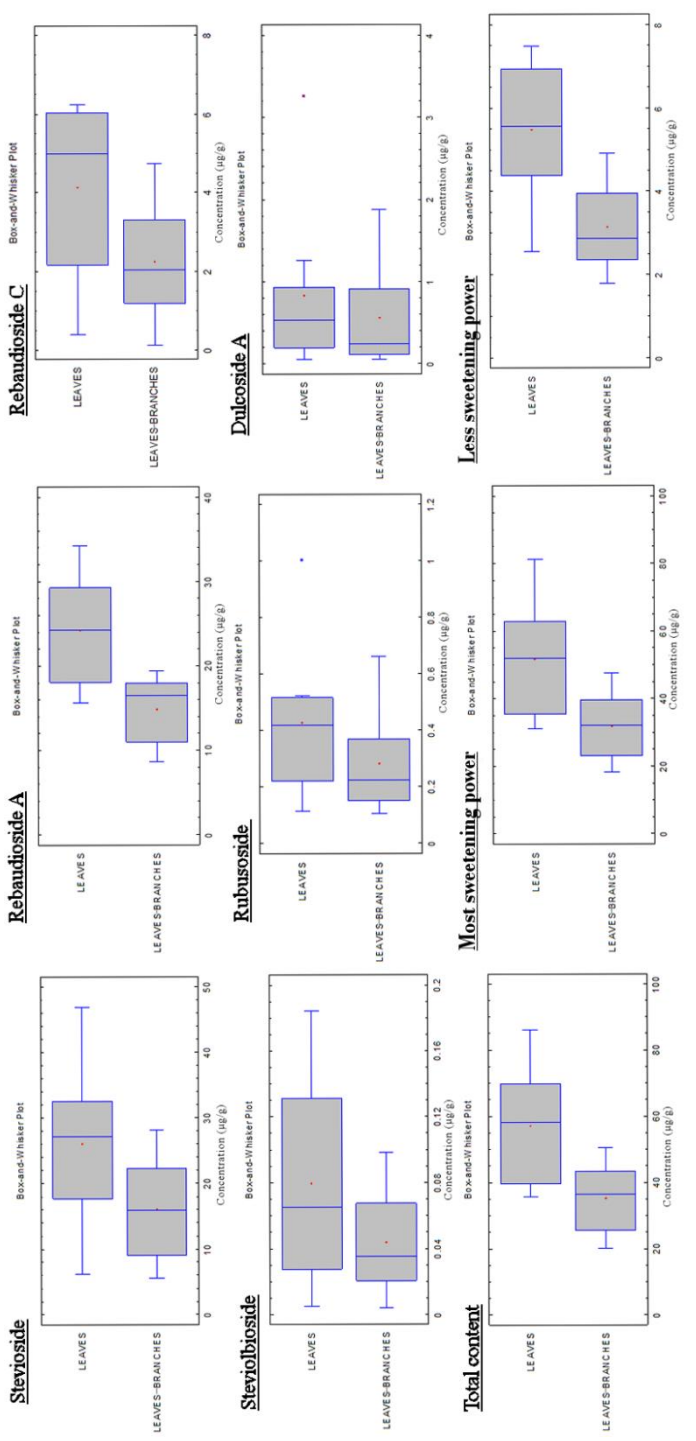
		Stevioside		Rebaudioside A		Rebaudioside B		Rebaudioside C		Rebaudioside D		Steviolbioside		Rubusoside		Dulcoside A	
		µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%
FIELD 1	L	20447 ± 265	43.24	20311 ± 303	42.96	523 ± 16	1.11	4387 ± 76	9.28	1117 ± 8	2.36	34.3 ± 0.9	0.07	232 ± 2	0.49	231 ± 2	0.49
	L+B	18164 ± 126	43.67	18069 ± 53	43.45	108.9 ± 9.7	0.26	3709 ± 43	8.92	1042 ± 9	2.51	26.2 ± 1.6	0.06	251 ± 7	0.60	219 ± 4	0.53
FIELD 2	L	6227 ± 586	16.64	21343 ± 1083	55.40	649 ± 80	1.74	6042 ± 380	16.15	3592 ± 362	9.60	5.0 ± 1.5	0.01	115.5 ± 10.6	0.31	54.5 ± 3.8	0.15
	L+B	5530 ± 51	17.22	17822 ± 364	55.49	645 ± 80	2.01	4750 ± 20	14.79	3210 ± 32	10.00	4.1 ± 1.4	0.01	105.8 ± 1.8	0.33	51.4 ± 2.3	0.16
FIELD 3	L	46896 ± 231	54.49	34295 ± 852	39.85	0.0 ± 0.0	0.00	401 ± 2	0.47	24.4 ± 2.3	0.03	183.9 ± 2.6	0.21	1003 ± 65	1.17	3263 ± 22	3.79
	L+B	28207 ± 276	55.95	19445 ± 524	38.57	0.0 ± 0.0	0.00	122.5 ± 2.0	0.24	0.0 ± 0.0	0.00	98.4 ± 0.6	0.20	661 ± 20	1.31	1881 ± 26	3.73
FIELD 4	L	31196 ± 30	45.20	29114 ± 127	42.18	139.6 ± 7.7	0.20	6250 ± 93	9.05	1100 ± 18	1.59	146.7 ± 11.7	0.21	465 ± 18	0.67	614 ± 10	0.89
	L+B	13805 ± 38	46.35	12522 ± 142	42.04	48.7 ± 4.1	0.16	2412 ± 48	8.10	474 ± 20	1.59	56.3 ± 6.9	0.19	201 ± 3	0.67	264 ± 5	0.89
FIELD 5	L	23109 ± 94	55.44	15885 ± 133	38.11	0.0 ± 0.0	0.00	848 ± 6	2.03	142.3 ± 8.2	0.34	73.1 ± 1.0	0.18	372 ± 3	0.89	1256 ± 7	3.01
	L+B	24674 ± 147	55.06	17170 ± 103	38.32	27.6 ± 8	0.06	915 ± 6	2.04	138.9 ± 5.7	0.31	78.2 ± 3.6	0.17	370 ± 10	0.83	1435 ± 13	3.20

Cont. Supplementary Table 4

FIELD 6	**L**	15023 ± 63	42.20	15608 ± 230	43.85	100.7 ± 9.0	0.28	3499.2 ± 43.5	9.83	971 ± 7	2.73	20.6 ± 0.6	0.06	211 ± 11	0.59	163.5 ± 3.9	0.46
	L+B	9062 ± 49	42.72	9415 ± 27	44.38	122.3 ± 7.0	0.58	1685.4 ± 51.9	7.94	657 ± 7	3.10	14.9 ± 1.0	0.07	158.1 ± 4.3	0.75	98.4 ± 2.5	0.46
FIELD 7	**L**	31698 ± 158	45.50	29405 ± 60	42.20	285.5 ± 15.2	0.41	6019.6 ± 68.5	8.64	1147 ± 11	1.65	115.7 ± 1.1	0.17	523 ± 23	0.75	480 ± 8	0.69
	L+B	9176 ± 226	45.69	8662 ± 114	43.13	105.4 ± 6.1	0.52	1462.0 ± 64.1	7.28	362 ± 11	1.80	33.5 ± 1.5	0.17	144.1 ± 2.3	0.72	136.8 ± 7.0	0.68



Supplementary Figure 1. Scan spectra of stevioside (1) and rebaudioside A (2) both at 2 µg/mL, and dulcoside A (3) at 5 µg/mL.



Supplementary Figure 2. Box and Whisker plots comparing the significantly different (*p*-value <0.05) concentration of steviol glycosides and their sum as a function of their sweetener power (µg/g) in Stevia plants by sampling leaves or leaves+branches.

CAPÍTULO VI

Dual method for characterization and quantitation of the phenolic fraction from Stevia leaves by LC–QqQ MS/MS

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Dual method for characterization and quantitation of phenolic fraction from Stevia leaves by LC−QqQ MS/MS

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Dual method for characterization and quantitation of phenolic fraction from Stevia leaves by LC−QqQ MS/MS

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ABSTRACT

Stevia is a currently well-known plant thanks to its sweet taste in which only the steviosides (sweet compounds) had so far been investigated in depth, despite the healthy properties of this plant cannot be attributed to its sweet components. In this research, a dual method based on LC–MS/MS by using a triple quadrupole detector was developed for characterization and quantitation of phenolic compounds in extracts from Stevia leaves. The study of the ionization parameters for the target analytes demanded for a dual method for independent analysis of caffeoylquinic acids and flavonoids. The MS/MS method was based on the selected reaction monitoring approach. The dual method led to the design of a calibration study that provided ranges of 0.5−25 ng/mL and 1−50 ng/mL as limits of detection and quantitation, respectively, and within-day and between-days variability lower than 12.5% and 22.0%, respectively. The target compounds were quantified in extracts from leaves of five different Stevia genetic varieties, finding significantly high concentrations of caffeoylquinic acids and quercetin glycosides that were at the level of mg/g of dry leaves. The results were used to evaluate the similarity among the varieties and also to study the correlation among the target compounds.

Keywords Stevia leaves; Quantitation; Triple quadrupole; Phenolic compounds; Caffeoylquinic acids; Flavonoids.

1. Introduction

Stevia is a schrub of the Asteraceae family native to South America, especially to Northeast of Paraguay [1]. Nowadays, the well-known sweet taste of Stevia leaves has turned this plant into a powerful alternative to artificial sweeteners and has spread Stevia crops to other regions of the world such as Canada, Asia and Europe [2]. The Stevia sweet taste is due to steviol glycosides such as rebaudioside A and stevioside as the most important; the wide research on them has promoted their approval as food additives in many countries worldwide. As an example, the European Food Safety Authority (EFSA) issued a positive scientific opinion on their safety and established their acceptable daily intake [3]. For these reasons, a wide number of studies were focused on quantitation of steviol glycosides in Stevia leaves, such as our previous work, where a method based on LC–MS/MS with a triple quadrupole detector was used to quantify 8 of these compounds in Stevia cultivated in the South of Spain [4].

Apart from steviol glycosides, Stevia is also rich in phenolic compounds, especially in caffeoylquinic acids. Phenols —metabolites widely distributed in the plant kingdom— are well known thanks to their beneficial properties for the organism, as recently reviewed by Roleira et al. [5]. Concerning caffeoylquinic acids, several authors have studied the effect in the organism of these compounds extracted from leaves of different plants, and to which antimutagenic [6], neuroprotective [7], antiviral [8], and antibacterial [9] properties have been attributed.

Several studies have focused on phenols composition in Stevia plants; however, most of them involved overall determination by simple methods as those based on derivatization with the Folin-Ciocalteu reagent, ferric reducing antioxidant power (FRAP), reaction with 2,2-diphenyl-1-picrylhydrazyl (DPPH) or radical scavenging activity [10–15]. These studies only provided data on the total concentration of phenols and they did not report information about the

nature and concentration of individual compounds. The first study focused on individual phenols in Stevia leaves was carried out by Karaköse et al., who characterized 24 hydroxycinnamate derivatives —mainly caffeoylquinic acids— using LC−MS^n with an ion-trap spectrometer, but only quantified 3 monocaffeoylquinic and 3 dicaffeoylquinic acids in methanolic extracts of Stevia leaves [16]. A recent study from the same authors completed the characterization of 15 flavonoids, but in this case they only quantified apigenin, kaempferol, luteolin and quercetin, apart from the caffeoylquinic acids previously quantified [17]. In contrast, the most comprehensive study of this family in Stevia was carried out by the authors of the present article, where the polar extract of Stevia leaves was characterized by LC−QTOF MS/MS [18]. In that study a total of 89 compounds distributed in 12 families were tentatively identified with special emphasis on caffeic acid and derivatives, and flavonoids. The results of the study suggested a significant concentration of these compounds in Stevia leaves, a fact that (together with the properties attributed to them) encouraged their quantitation. Thus, the primary aim of the research here presented was to develop an analytical method able to characterize quantitatively the phenolic fraction of Stevia leaves. Special attention has been paid to caffeoylquinic acids and flavonoids due to their concentration in the samples. For this purpose, a dual method has been developed for analysis of polar extracts from Stevia leaves by LC−MS/MS.

2. Experimental

2.1. Samples

Leaves of *Stevia rebaudiana bertoni* from different varieties and commercial crossbreedings were used for this study. Samples from the varieties *Eirete*×*Morita*, *Katupyry*, *Eirete*, *Criolla* and *Candy* were collected in June 2016 and the leaves were oven-dried for 3 days at 30 ºC. Subsequently, the dried leaves were chopped until

reaching a homogeneous particle size.

2.2. Standards and reagents

3-Caffeoylquinic, 5-caffeoylquinic, 1,3-dicaffeoylquinic, quinic, shikimic, 2-coumaric and 3-coumaric acids were purchased from Extrasynthese (Genay, France). 4-Caffeoylquinic, 3,4-dicaffeoylquinic, 3,5-dicaffeoylquinic, 4,5-dicaffeoylquinic, caffeic, sinapic, 4-coumaric, 3-methylcatechol, 4-methylcatechol, apigenin, apigenin-7-glucoside, kaempferol, kaempferol-7-neohesperidoside, luteolin, luteolin-7-glucoside, orientin, homoorientin, quercetin, quercetin-3-glucoside, quercitrin, rutin and quercetin-3-arabinoside were from Sigma–Aldrich (St. Louis, USA). All reagents were of analytical grade or higher. Deionized water (18 MΩ·cm) was obtained by a Milli-Q water purification system from a Millipore (Bedfore, MA, USA) Milli-Q plus system and LC-grade methanol was from Panreac (Barcelona, Spain).

2.3. Apparatus and instruments

A vortex shaker IKA MS3 digital (Staufen, Germany) was used for extraction. An Agilent 1200 Series LC system from Agilent Technologies (Palo Alto, USA) with a 6460 Triple Quad LC–MS detector equipped with a Jet Stream Technology electrospray ion source, also from Agilent, was used to carry out the analyses of the extracts. A Mediterranea Sea C18 analytical column (5 µm, 15 × 0.46 cm) and a C18 precolumn (5 µm, 4 × 3 mm) from Tecknokroma (Barcelona, Spain) were selected for the chromatographic separation.

2.4. Extraction step

Phenolic compounds were extracted from the Stevia dry leaves by maceration for 30 min using 15 mL of a methanol–water (75:25, v/v) mixture as extractant. The extract was isolated and the resulting residue was subjected to two additional extraction steps. The combination of the extracts led to approximately 45 mL of extract, which was filtered by a 20-µm filter and stored at –20 ^{o}C until analysis.

2.5. LC−QqQ MS/MS methods

Two analytical methods were developed for the analysis of the target compounds. In both methods, deionized water with 0.1% of formic acid (A) and methanol with 0.1% of formic acid (B) were used as mobile phases. In the first method, the gradient was as follows: start with 0% B, change from 0% to 20% B in 10 min, change from 20% to 46% in 8 min, keep this percentage of B for 7 min, change from 46% to 60% in 5 min, and then constant 60% B for 2 min more. In the second method, the gradient was: start with 20% B, change to 60% B in 7 min, change to 64% in 3 min, keep this percentage of B for 5 min, change to 75% in 7 min, and then constant 75% B for 3 min more. In both methods, a post-run of 5 min was programmed to equilibrate the column between analyses. The flow rate was constant at 0.7 mL/min and the injected volume was 10 µL. The column temperature was set at 15 ºC.

High-purity nitrogen (99.999%) as collision gas was used in the triple quadrupole MS. Determination of the compounds was performed by ESI-MS/MS in SRM mode. In both methods, the sheath gas flow and temperature were set at 12 mL/min and 400 ºC, respectively, and the pressure of the nebulizer was 45 psi. In the first method, the gas temperature was set at 250 ºC, and at 200 ºC in the second. The capillary voltage was established at 6000 and 1000 V and the nozzle voltage at 1000 and 500 V in the first and second method, respectively, for the negative ionization mode.

2.6. Quantitation of phenolic compounds

The calibration curves were run by plotting the peak area of each compound versus its nominal concentration in the multistandard solutions. For this purpose, 12 calibration levels were prepared in methanol with the target analytes at suited concentrations to define the calibration ranges and evaluate the sensitivity of the quantitation method by estimation of the limits of detection and quantitation (LODs and LOQs, respectively).

3. Results and discussion

3.1. Optimization of the SRM detection mode

The target compounds were selected on the basis of the review by Wölwer-Rieck [19], who described the phenolic composition of Stevia. Optimization of the MS/MS method was targeted at the main parameters involved in MS ionization and fragmentation to achieve the highest sensitivity in detection. The ionization operating conditions were studied by direct injection of individual standard solutions of each analyte at 2 μg/mL using the negative ionization mode. Thus, the $[M–H]^-$ ion was generated as the most intense precursor ion in all the compounds under study and, therefore, this ion was selected for MS/MS fragmentation. The MS/MS parameters were optimized for efficient isolation of the precursor ions and their sensitive and selective fragmentations. Voltage of the first quadrupole (Q1 voltage) for an efficient filtration of the precursor ions, collision energy, and selected product ions for the quantitation transitions were the variables under study (see Table 1). As can be seen, phenolic compounds presented an optimum Q1 voltage below 100 V, excluding quinic acid; while flavonoids required a voltage above 100 V in all cases.

Concerning MS fragmentation, it should be highlighted that some of the phenols under study (viz., 2-, 4-coumaric acid, shikimic acid) provided optimum collision energy below 10 eV since the precursors were completely fragmented and not detected at higher collision energy values. The caffeoylquinic acids presented a characteristic fragmentation, as can be seen in Fig. 1. The 3- and 5-caffeoylquinic acids presented one characteristic fragment at 191.0 *m/z* resulting from the loss of the caffeic moiety, while the 4- isomer did not show a preferred fragmentation being the *m/z* 179.1, generated from the loss of the quinic moiety, the most intense product ion. Concerning dicaffeoylquinic acids, the isomers 1,3- 3,4- and 3,5- provided the fragment 353.1 as the most intense since it is generated by the loss of one caffeic moiety. Nevertheless, the 4,5-dicaffeoylquinic acid presented a higher

fragmentation level providing the *m/z* 179.1 fragment as the most intense due to the loss of caffeic and quinic acid moieties.

After optimization of the fragmentation parameters, the influence of the ESI source parameters on the LC–MS/MS sensitivity was studied (see Supplementary Table 1). Particular differences were found in the response of the target analytes according to two parameters involved in the ionization process. These were the capillary voltage and the nozzle voltage. Thus, some of the target compounds, especially caffeoylquinic acids, provided the best response with high values of capillary voltage, 6000 V. On the other hand, the monitored flavonoids reported the highest signals when this parameter was set at 1000 V. A similar trend was observed with the nozzle voltage as the obtained results demanded a higher value for caffeoylquinic acids as compared to flavonoids, 1000 V versus 500 V. The influence of both parameters on some representative caffeoylquinic acids and flavonoids can be checked in Fig. 2. It is worth mentioning that ESI offers the possibility of controlling the degree of ion excitation by changing the potential between the capillary or nozzle and the skimmer in the ESI source [20–22]. This effect is strongly associated to the structure of the molecules and, in this context, clear differences are observed for caffeoylquinic acids and flavonoids, the latter characterized by a rigid structure. The differences observed in the two voltages supported the need to develop two independent methods for analysis of phenolic compounds in Stevia leaves in order to maximize sensitivity. Thus, the determination of caffeoylquinic acids, quinic, caffeic, shikimic, sinapic, cinnamic, 2-coumaric, 3-coumaric and 4-coumaric acids, and 3-methylcatechol and 4-methylcatechol was carried out with the first method, while flavonoids were determined by in the second method.

Table 1. Parameters of the dual LC–MS/MS method for quantitative analysis of the target compounds and their calibration parameters, LOD and LOQ.

	Compound	Retention time (min)	Transition	Q1 Voltaje (V)	Collision energy (eV)	Equation	R^2	LOD (ng/mL)	LOQ (ng/mL)
Method 1									
Quinic, caffeic and caffeoylquinic acids	Quinic acid	3.2	191.1 → 84.9	130	20	y = 5162.6x - 84.005	0.9998	2.5	5
	5-Caffeoylquinic acid	12.6	353.1 → 191.1	95	15	y = 52572x - 2827.9	0.9917	5	10
	4-Caffeoylquinic acid	15.1	353.1 → 179.1	95	10	y = 24902x + 709.65	0.9996	10	25
	3-Caffeoylquinic acid	15.3	353.1 → 191.1	90	15	y = 74963x - 1638.1	0.9968	5	10
	1,3-Dicaffeoylquinic acid	16.1	515.1 → 353.1	95	15	y = 33841x + 137.98	0.9997	5	10
	Caffeic acid	16.6	179.1 → 135.1	85	10	y = 43768x + 1253	0.9998	25	50
	4,5-Dicaffeoylquinic acid	19.9	515.1 → 179.1	95	25	y = 5358.3x - 194.52	0.9992	25	50
	3,5-Dicaffeoylquinic acid	20.8	515.1 → 353.1	90	10	y = 40189x - 913.42	0.9995	10	25
	3,4-Dicaffeoylquinic acid	24.9	515.1 → 353.1	95	15	y = 35324x - 1801.1	0.9976	10	25
Phenolic compounds	Shikimic acid	4.6	173.1 → 93.2	80	7	y = 5428.9x + 167.77	0.9996	10	25
	4-Methylcatechol	16.3	123.1 → 108.0	80	12	y = 1398.4x + 100.36	0.9979	10	25
	3-Methylcatechol	17.1	123.1 → 108.0	80	16	y = 1962.4x + 91.585	0.9988	10	25
	4-Coumaric acid	19.1	163.1 → 119.1	80	7	y = 45418x + 1855.1	0.9985	2.5	5
	Sinapic acid	19.5	223.1 → 193.1	85	10	y = 6015.1x + 60.926	0.9998	5	10
	3-Coumaric acid	20.6	163.1 → 119.1	75	10	y = 23201x + 627.26	0.9995	5	10
	2-Coumaric acid	22.5	163.1 → 119.1	75	5	y = 43319x + 512.9	0.9996	2.5	5

Cont. Table 1

Method 2									
Flavonoids	Orientin	10.2	447.1 → 327.0	180	20	y = 103314x + 2195.1	0.9995	1	2.5
	Homoorientin	10.5	447.1 → 357.0	125	20	y = 71403x + 9578.3	0.9841	1	2.5
	Luteolin-7-glucoside	11.4	447.1 → 285.1	210	20	y = 180325x - 246.66	0.9991	1	2.5
	Rutin	11.7	609.1 → 300.0	130	40	y = 23201x + 627.26	0.9995	1	2.5
	Kaempferol-7-neohesperidoside	11.7	629.1 → 593.1	110	15	y = 7737.8x - 47.322	0.9987	5	10
	Quercetin-3-glucoside	11.8	463.1 → 300.0	120	25	y = 69540x + 4101	0.9964	1	2.5
	Apigenin-7-glucoside	12.3	431.1 → 268.1	205	30	y = 257429x - 185.73	0.9994	0.5	1
	Quercetin-3-arabinoside	12.4	433.1 → 300.0	115	20	y = 45418x + 1855.1	0.9985	2.5	5
	Quercitrin	12.7	447.1 → 300.0	165	20	y = 43319x + 512.9	0.9996	1	2.5
	Quercetin	14.8	301.0 → 107.1	120	20	y = 12097x - 485.06	0.9985	25	50
	Luteolin	16.0	285.1 → 133.0	150	25	y = 76313x - 140.79	0.9965	1	2.5
	Kaempferol	17.7	285.1 → 93.0	120	30	y = 5281.8x - 61.497	0.9999	25	50
	Apigenin	18.7	269.1 → 117.1	120	25	y = 47526x - 87.633	0.9952	1	2.5

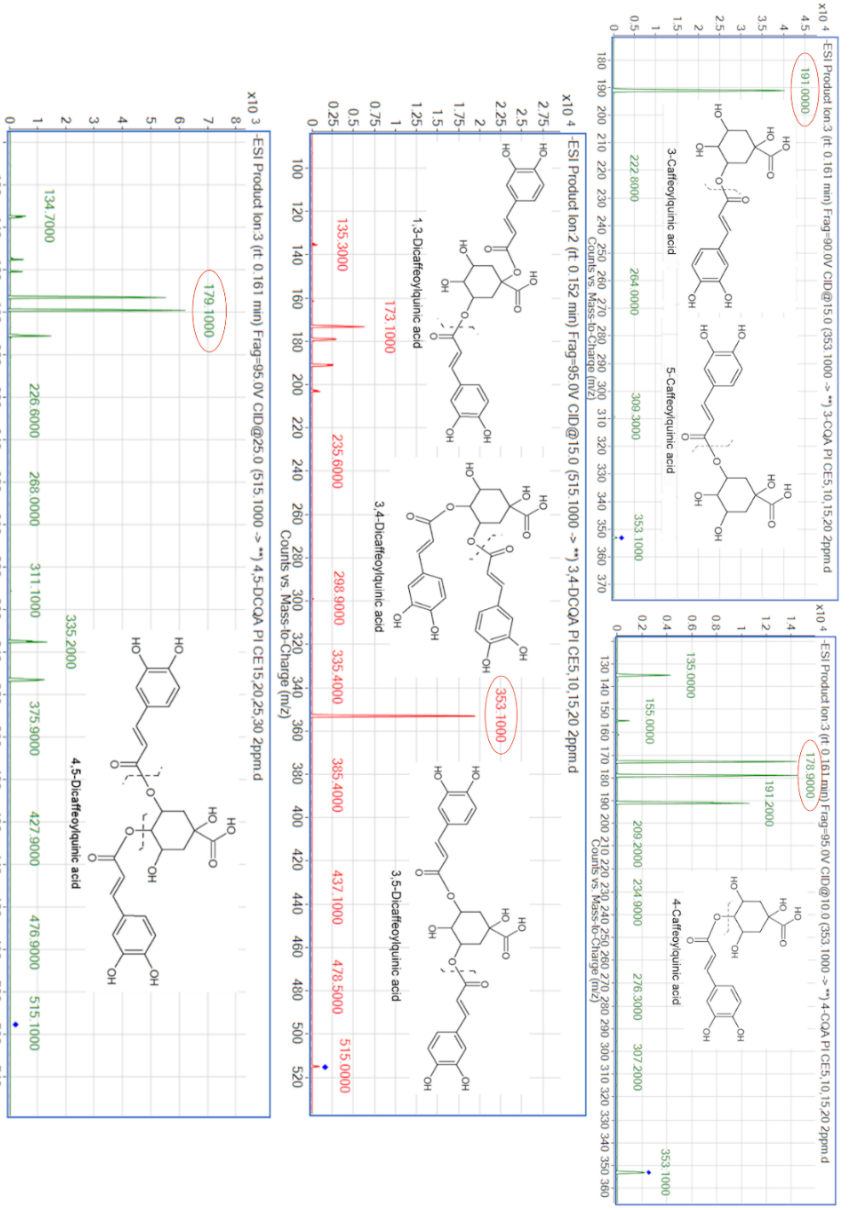

Figure 1. MS/MS spectra and fragmentation pattern from each caffeoylquinic acid.

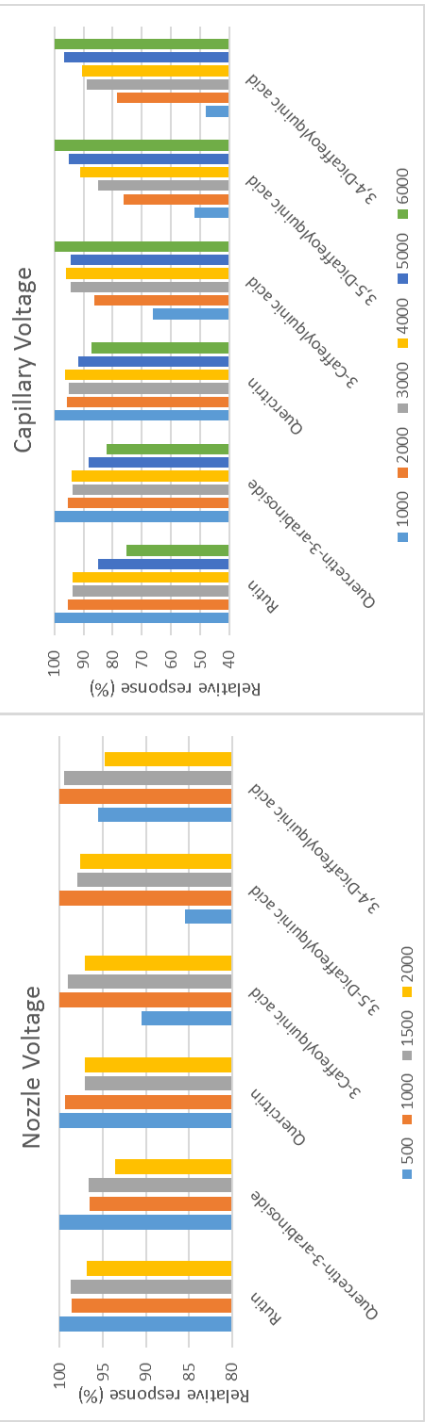

Figure 2. Relative response of the main flavonoids and caffeoylquininc acids found in Stevia leaves according to the nozzle and capillary voltages. Relative responses were estimated in percentage by setting the maximum signal obtained for each phenol as reference.

3.2. Calibration models and evaluation of sensitivity and precision

A linear regression model was applied to generate a calibration curve in the concentration range from LOQ to 5 µg/mL for all the target compounds. The regression coefficients (R^2) of the calibration models were higher than 0.98, indicative of a good fitting (see Table 1). The sensitivity of the dual method was evaluated by estimation of the limits of detection (LODs) and quantitation (LOQs) for each analyte by injecting dilution series of two multistandard solutions (one multistandard for each set of compounds) to obtain the concentration which provided signals three and ten times the background noise, respectively (see Table 1). The LOQs for caffeoylquinic acids were within the range 10−50 ng/mL, while the LODs were within 5−10 ng/mL. The rest of the phenolic compounds presented wider LOQ and LOD ranges: within 5−50 and 2.5−25 ng/mL, respectively. On the other hand, the target flavonoids provided the lowest LOQ and LOD values, within 1−50 and 0.5−25 ng/mL, respectively.

The precision of the dual method was evaluated by calculation of the within-day variability and between-days variability both expressed as RSD. For this purpose, a single experimental set-up with triplicate analysis per day was carried out for seven days with a pool from the extracts of all the Stevia cultivars under study. The results, listed in Table 2, show that the between-days variability was below 22% in all cases, while the within-day variability was below 9%, excluding apigenin, apigenin-7-glucoside and kaempferol that reported 11.1, 11.0 and 12.3%, respectively.

Table 2. Precision parameters for determination of phenolic compounds in Stevia leaves by the proposed dual method.

Compound	Within-day variability (%)	Between-days variability (%)
3-Caffeoylquinic acid	1.8	5.5
4-Caffeoylquinic acid	1.6	19.4
5-Caffeoylquinic acid	3.0	18.9
3,4-Dicaffeoylquinic acid	5.4	18.0
3,5-Dicaffeoylquinic acid	5.5	16.5
4,5-Dicaffeoylquinic acid	1.7	11.3
Quinic acid	3.5	20.8
Shikimic acid	4.1	20.7
Apigenin	11.1	19.9
Apigenin-7-glucoside	11.0	20.0
Kaempferol	12.3	12.9
Kaempferol-7-neohesperidoside	8.6	11.8
Luteolin	8.7	17.0
Luteolin-7-glucoside	6.9	22.0
Quercetin	5.4	12.9
Quercetin-3-glucoside	6.2	16.0
Quercitrin	2.5	21.0
Rutin	4.5	19.6
Quercetin-3-arabinoside	2.9	19.7

3.3. Optimization of the extraction procedure

The extractant and the number of extraction steps needed for complete isolation of the target compounds were optimized. Firstly, methanol−water mixtures at different percentages were assayed to obtain the highest extraction yield. A pool with all the Stevia varieties was used as raw material and the compounds were extracted with 0, 25, 50, 75 and 100% methanol in deionized water. The extracts thus obtained were analyzed with the proposed dual method and the resulting chromatograms are shown in Supplementary Fig. 1. The

chromatogram corresponding to the main flavonoids (A) shows that 75 and 50% of methanol provided the highest signals for the major chromatographic peaks. The same behavior was found for the chromatogram of caffeoylquinic acids, but in this case the extracts obtained with 75% of methanol clearly provided a higher intensity in most of the peaks as compared to 50%. For these reasons, 75% was selected as the optimum percentage of methanol in water for extraction of all target compounds.

To confirm extraction quantitativeness, three consecutive re-extractions on the same solid residue were carried out. The extracts thus obtained were analyzed with the dual method and the peaks from 3-caffeoylquinic, 3,5-dicaffeoylquinic and 3,4-dicaffeoylquinic acids were used for comparison of the efficiency of each extraction step since they provided the highest chromatographic signal. The peak area of these metabolites obtained by analysis of the four extracts were compared (see Supplementary Fig. 2) resulting that the third re-extraction provided a no significant concentration of 3-caffeoylquinic, 3,5-dicaffeoylquinic and 3,4-dicaffeoylquinic as compared to that obtained in the first extract. Thus, only the first and the two subsequent extractions were taken into account for quantitation of the target compounds.

3.4. Analysis of extracts from leaves of Stevia cultivars

The proposed dual LC−MS/MS method was applied to the analysis of the target compounds in the Stevia varieties under study. Firstly, it should be mentioned that 1,3-dicaffeoylquinic, sinapic, 2-, 3-, 4-coumaric, 3-, 4-methylcatechol, orientin and homoorientin were not detected in the samples. Supplementary Table 2 lists the concentration of the target compounds found in the analyzed samples expressed as mg/g or as μg/g of dry leaves. Caffeic acid provided a signal lower than the LOQ; therefore, it could not be quantified in the samples despite it was detected in several cultivars. By contrast, caffeoylquinic acids provided the highest concentration as compared to most of the flavonoids,

reaching more than 100 mg/g of dry leaves for 3,5- and 4,5-dicaffeoylquinic acids in the *Eirete*×*Morita* cultivar. Concerning monocaffeoylquinic acids, the 3-isomer was the most concentrated in all samples, while isomers 3,4- and 3,5- provided the highest concentration for dicaffeoylquinic acids in all varieties, excluding *Eirete*×*Morita*, where the above mentioned were the most concentrated isomers (see Fig. 3). Within flavonoids, kaempferol was detected in all samples, but it could only be quantified in the *Candy* cultivar, while kaempferol-7-neohespesridoside was detected under its LOQ for *Eirete*×*Morita* and *Criolla* varieties. On the other hand, the glycosylated forms of quercetin (quercetin-3-glucoside, quercitrin, rutin and quercetin-3-arabinoside) provided remarkable high concentrations in all the samples, reaching more than 13 mg/g of dry leaves for quercitrin in the *Eirete* cultivar (see Fig. 3).

A clustering analysis with a hierarchical algorithm was carried out to study the similarity among the Stevia cultivars as a function of the concentration of the target compounds in them. The resulting dendrogram (Supplementary Fig. 3) showed that *Candy* and *Criolla* varieties presented the highest similarity, while the variety *Eirete*×*Morita* was the most different. Concerning the target compounds, caffeoylquinic acids exhibited a similar concentration range in all the varieties, being slightly higher in the variety *Eirete*×*Morita*. The most significant differences were found in apigenin, kaempferol, luteolin and their glycoside forms; however, their concentration were so small that their statistical weights should not be taken into account to explain the similarity among varieties as compared to caffeoylquinic acids and quercetin and its glycosides. The last group, especially rutin and quercetin-3-arabinoside, presented higher concentrations in leaves from *Eirete*×*Morita* and *Eirete* Stevia cultivars, which clearly differentiate these varieties with respect to the others.

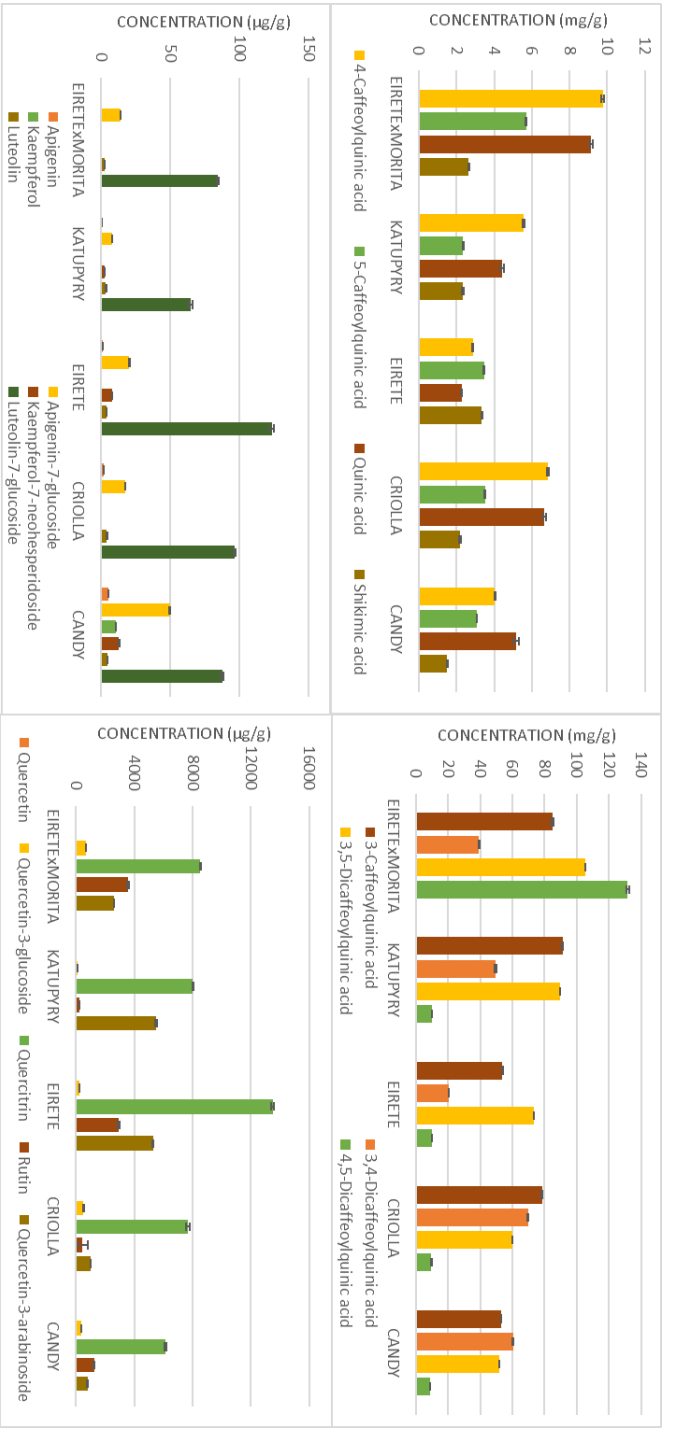

Figure 3. Concentration of the target phenols found in Stevia leaves from the varieties under study.

Finally, a correlation test was applied to the results to detect a potential relationship in the concentration of the target compounds in the samples. Supplementary Table 3 shows the correlations found between different compounds with a *p*-value below 0.05. All the correlations provided an R value above 0.85, which means that there is a strong relationship between the concentration of these compounds. 4-Caffeoylquinic acid is strongly related to quinic acid and the positive value of R indicates that this relationship is direct. Based on this fact, it can be thought that 4-caffeoylquinic acid is the monocaffeoylquinic isomer firstly formed by addition of caffeic acid to quinic acid since its concentration is directly related to quinic acid concentration. Within this family, it should also be highlighted the direct relationship between 5-caffeoylquinic and 4,5-dicaffeoylquinic acids, which could involve the formation of the dicaffeoylquinic from the monocaffeoylquinic acid.

Concerning flavonoids, apigenin resulted to be related to apigenin-7-glucoside and kaempferol by a similar behavior, which have sense since apigenin is the precursor of kaempferol in the flavone and flavonol biosynthesis pathways, and apigenin-7-glucoside is formed by glucosylation of the aglycon. Quercetin and rutin have opposite behaviors, suggesting a formation of rutin at the expenses of quercetin in the Stevia leaves. Finally, the unknown relationship between 5-caffeoylquinic acid and quercetin-3-glucoside; 3,5-dicaffeoylquinic acid and luteolin; and shikimic acid and quercitrin could be attributed to unknown metabolic pathways that lead these compounds to keep a proportional concentration in Stevia leaves.

4. Conclusions

Two complementary LC−ESI−MS/MS methods were developed for quantitation of phenolic compounds in Stevia leaves to apply the optimum

fragmentation conditions depending on the features of the target metabolites. The dual method allowed determination of 33 compounds in the samples, with ranges of 0.5−25 ng/mL and 1−50 ng/mL for limits of detection and quantitation, respectively; and variability within-day and between-days (expressed as RSD) lower than 12.5% and 22.0%, respectively. The proposed methods were applied to the quantitation of phenolic compounds in Stevia leaves from different varieties, thus correlating the behavior of some of the target compounds, which can be linked to the mechanisms and pathways involved in the synthesis of some of them.

Acknowledgements

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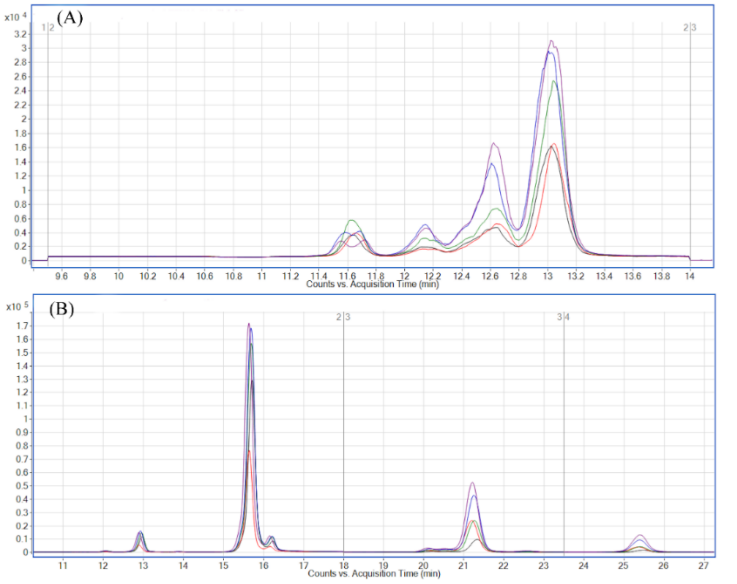
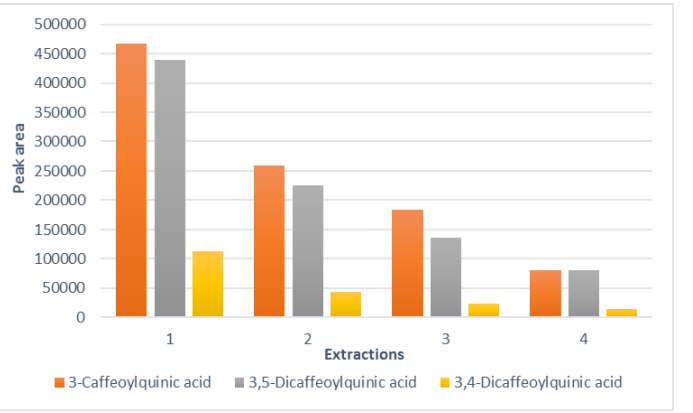
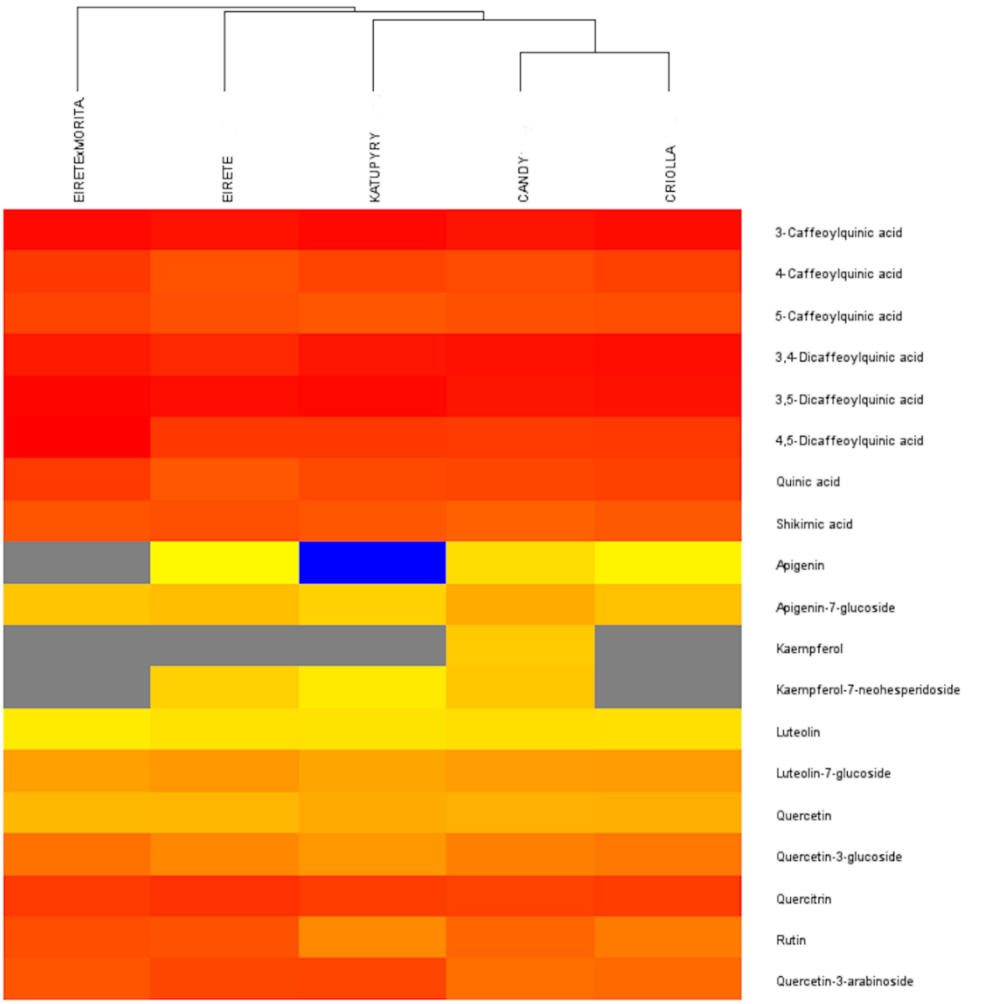
Supplementary information



Supplementary Figure 1. Extracted ion chromatograms of the main flavonoids (A) and caffeoylquinic acids (B) from the Stevia extracts obtained with 0 (black), 25 (green), 50 (blue), 75 (purple) and 100% (red) of methanol in deionized water.



Supplementary Figure 2. Peak areas from 3-caffeoylquinic, 3,5-dicaffeoylquinic and 3,4-dicaffeoylquinic acids by LC–MS/MS analysis of Stevia extracts obtained by a first extraction (1) and after three consecutive re-extractions (2, 3 and 4) of Stevia leaves.



Supplementary Figure 3. Dendrogram obtained by cluster analysis to show similarities among the Stevia cultivars according to the phenolic concentration.

Supplementary Table 1. Studied ranges for ESI source parameters and the optimum values for both methods.

Parameter	Tested range	Optimum value Method 1	Optimum value Method 2
Gas temperature (°C)	100–400	250	200
Sheath gas temperature (°C)	100−425	400	400
Capillary voltage (V)	0−6000	6000	1000
Nozzle voltage (V)	0−2000	1000	500

Supplementary Table 2. Concentration of the target compounds in leaves from different Stevia varieties.

Compound	Eirete× Morita	Katupyry	Eirete	Criolla	Candy
	Concentration (mg/g of dry leaves)				
3-Caffeoylquinic acid	85.0 ± 0.7	91.2 ± 0.8	53.8 ± 0.8	78.3 ± 0.9	53.0 ± 0.5
4-Caffeoylquinic acid	9.8 ± 0.1	5.6 ± 0.1	2.9 ± 0.1	6.9 ± 0.1	4.0 ± 0.1
5-Caffeoylquinic acid	5.7 ± 0.1	2.3 ± 0.0	3.5 ± 0.1	3.5 ± 0.1	3.1 ± 0.0
3,4-Dicaffeoylquinic acid	39.3 ± 0.5	49.7 ± 1.1	20.5 ± 0.5	69.6 ± 0.7	60.4 ± 0.5
3,5-Dicaffeoylquinic acid	105 ± 1	89.4 ± 1.1	73.2 ± 0.6	60.2 ± 0.6	52.1 ± 0.4
4,5-Dicaffeoylquinic acid	132 ± 1	10.1 ± 0.2	9.9 ± 0.2	9.6 ± 0.3	8.7 ± 0.3
Caffeic acid	UNDER LOQ	UNDER LOQ	UNDER LOQ	UNDER LOQ	UNDER LOQ
Quinic acid	9.2 ± 0.1	4.4 ± 0.2	2.3 ± 0.0	6.7 ± 0.1	5.2 ± 0.3
Shikimic acid	2.6 ± 0.1	2.3 ± 0.1	3.3 ± 0.0	2.2 ± 0.1	1.5 ± 0.0
	Concentration (µg/g of dry leaves)				
Apigenin	UNDER LOQ	0.8 ± 0.0	1.3 ± 0.0	1.7 ± 0.1	5.1 ± 0.1
Apigenin-7-glucoside	14.1 ± 0.5	8.0 ± 03	20.4 ± 0.5	17.5 ± 0.5	49.6 ± 1.1
Kaempferol	UNDER LOQ	UNDER LOQ	UNDER LOQ	UNDER LOQ	10.9 ± 0.3
Kaempferol-7-neohesperidoside	UNDER LOQ	2.7 ± 0.3	8.0 ± 0.6	UNDER LOQ	13.1 ± 0.7
Luteolin	2.5 ± 0.1	3.6 ± 0.1	3.8 ± 0.1	4.2 ± 0.2	4.6 ± 0.1
Luteolin-7-glucoside	84.5 ± 1.6	64.7 ± 2.3	123 ± 3	96.7 ± 1.5	87.6 ± 2.1
Quercetin	26.8 ± 0.1	51.2 ± 1.5	28.3 ± 0.4	41.2 ± 0.5	39.3 ± 0.8
Quercetin-3-glucoside	672 ± 8	124 ± 5	250 ± 4	522 ± 10	348 ± 7
Quercitrin	8516 ± 68	8016 ± 101	13476 ± 230	7684 ± 290	6121 ± 151
Rutin	3582 ± 128	237 ± 13	2951 ± 143	420 ± 148	1244 ± 62
Quercetin-3-arabinoside	2631 ± 190	5477 ± 119	5290 ± 84	1021 ± 28	784 ± 18

Supplementary Table 3. Pearson correlation analysis between target compounds after applying a correlation test.

	4,5-Dicaffeoylquinic acid		Quinic acid		Apigenin-7-glucoside		Kaempferol		Luteolin		Quercetin-3-glucoside		Quercitrin		Rutin	
	R	*p*-Value	R	*p*-Value	R	*p*-Value	R	*p*-Value	R	*p*-Value	R	*p*-Value	R	*p*-Value	R	*p*-Value
4-Caffeoylquinic acid																
5-Caffeoylquinic acid	0.926	0.023														
3,5-Dicaffeoylquinic acid									-0.969	0.006						
Shikimic acid													0.941	0.016		
Apigenin					0.953	0.011	0.947	0.014								
Apigenin-7-glucoside							0.958	0.010								
Quercetin															-0.944	0.015

PARTE C

Caracterización de la fracción volátil y polar de ajo fresco y negro: evolución durante la fermentación

La parte C de esta Memoria de Tesis está dedicada a uno de los sazonadores más utilizados en la cocina española y que cada vez se extiende a un número mayor de países. Las cualidades del ajo (como germicida, bactericida, antioxidante, protector cardiovascular, etc.) junto con los nuevos productos que se obtienen de su fermentación (ajo negro o fermentado) son los principales motores de la gran expansión actual del ajo.

La investigación realizada abarca tanto los compuestos volátiles del ajo fresco y fermentado como los no volátiles, muchos de ellos de enorme interés y poco o nada conocidos.

Los estudios de la fracción volátil se realizaron en todos los casos con un muestreador de espacio de cabeza conectado a un equipo GC–MS. El primer estudio se centró en 3 variedades de ajo fresco: blanco, morado y chino, según se recoge en el Capítulo VII. Se identificaron de forma tentativa 45 volátiles en ajo —17 de ellos identificados por primera vez. Todos ellos se clasificaron por primera vez y su concentración relativa se utilizó para evaluar las diferencias entre variedades, de las que se estudiaron los perfiles en función del tiempo de calentamiento.

El segundo estudio abarcó la comparación de la fracción volátil de ajo fresco con la de negro (ajo morado en este estudio), la evolución de esta fracción en ambos con el tiempo de calentamiento al que se someten en el espacio de cabeza, así como la evolución de los volátiles a lo largo del proceso de fermentación; tal como se recoge en el Capítulo VIII. El número de compuestos volátiles tentativamente identificados en ajo negro fue mayor que en ajo fresco; lo que pone de manifiesto la magnitud de los cambios producidos, en buena parte debidos a reacciones de Maillard. El seguimiento de la evolución de los volátiles durante la fermentación permitió delimitar los tiempos a los que se producen cambios significativos.

Un estudio imprescindible —realizado con un equipo LC–QTOF MS/MS— tras los anteriores es el global de los compuestos existentes en ajo fresco y en negro, su comparación, la evolución de los compuestos con la fermentación y la interpretación de las rutas seguidas en la transformación. Con estas premisas, se realizó la investigación que se recoge en el Capítulo IX, en la que se identificaron tentativamente y se clasificaron en familias 98 compuestos existentes en ajo fresco; composición que se comparó con la del ajo negro, concluyendo que el proceso de fermentación cambia considerablemente la composición de las diferentes familias que componen el ajo, especialmente los aminoácidos, los compuestos organosulfurados y los sacáridos. Las rutas metabólicas de los diferentes procesos en los que están implicados los compuestos especialmente afectados por la fermentación constituyen la investigación recogida en el Capítulo X.

CAPÍTULO VII

HS–GC–MS volatile profile of different varieties of garlic and their behavior under heating

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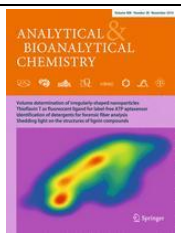

HS–GC−MS volatile profile of different varieties of garlic and their behavior under heating

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HS–GC−MS volatile profile of different varieties of garlic and their behavior under heating

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ABSTRACT

Garlic is one of the most used seasoning in the world whose health beneficial properties, mainly ascribed to organosulfur compounds, are shared with the rest of the *Allium* family. The fact that many of these compounds are volatile makes interesting the evaluation of the volatile profile of garlic. For this purpose, three garlic varieties —*White*, *Purple* and *Chinese*— cultivated in the South of Spain were analyzed by a method based on a headspace (HS) device coupled to a gas chromatograph and mass detector (HS–GC−MS). The main temperatures in the HS were optimized to achieve the highest concentration of volatiles. A total of 45 volatiles were tentatively identified (among them 17 were identified for the first time in garlic); then, all were classified also for the first time, and their relative concentration in three garlic varieties was used to evaluate differences among them and to study their profiles according to the heating time. *Chinese* garlic was found to be the richest variety in sulfur volatiles, while the three varieties presented a similar trend under preset heating times allowing differentiation between varieties and heating time using Principal Component Analysis.

Keywords Garlic varieties; Headspace; GC−MS; Sulfur volatiles; Flavor; Heating times

1. Introduction

Garlic (*Allium sativum*) is widely used in cuisines worldwide as seasoning, especially in Asia, Africa and Europe. Historically, garlic was already used by the Egyptians in several therapeutic formulas [1]. Also, Greeks and Romans used garlic as healing agent, which led to the expansion of its use to the whole Mediterranean region [2]. Spain assumed this historical heritage and became one of the major producers of garlic in the European Union, increasing its production to reach 173 million of tones of garlic in 2013 [3]. Research on garlic to explain its properties, known since antiquity, started in the second half of the 19th century when Louis Pasteur assessed its antibacterial properties [4]. More recent studies have reported that garlic extracts act as antioxidant and antimicrobial agents and also produce beneficial effects against cardiovascular diseases, as reviewed by Corzo-Martínez et al. [5]. Nowadays, attention has focused on the cancer preventive properties of garlic [6–10]. As examples, Jin et al. showed the protective association between garlic intake and lung cancer [11], while Huang et al. demonstrated the anti-proliferative effect on colon cancer cells of diallyl disulfide, one of the most characteristic compounds in garlic [12].

The beneficial properties of garlic have been related to the presence of organosulfur compounds, mainly thiosulfinates and sulfur volatiles, which are shared with the rest of the *Allium* family. Thiosulfinates, derivatives from S-alk(en)yl-L-cysteine, are unstable compounds and undergo chain reactions that transform them into volatiles [13], which are responsible for the characteristic garlic aroma and its potential anticancer activity, as demonstrated by Yang et al. and Seki et al. for diallyl disulfide and diallyl trisulfide [14, 15].

Methods for identification of volatile compounds in garlic have been supported on sample preparation procedures based on solid phase microextraction (SPME) or headspace separation (HS) prior to gas chromatography–mass spectrometry (GC–MS) [16–20]. Sample preparation

procedures proposed by Warren et al. for extraction of thiol volatiles were based on either in-needle or in-fiber derivatization with N-phenylmaleimide, which endowed the products with high selectivity [19]; while Lee et al. compared different procedures for extraction of volatiles (viz., steam distillation, simultaneous distillation and solvent extraction, solid-phase trapping solvent extraction and HS–SPME), resulting HS–SPME the most suitable because of the absence of solvent and the relatively low temperature that hindered degradation of thermolabile volatiles [21]. Kim et al. developed research to evaluate the content of volatile compounds in garlic subjected to different processing conditions: autoclaving, high temperature aging (black garlic), crushing and roasting at different temperatures. The resulting garlic products were analyzed by HS–SPME–GC−MS and 26 volatiles were identified and relatively quantified in the samples [18]. Finally, in a recent work by Radulović et al. 78 organosulfur compounds were detected in the essential oil of wild garlic using GC−FID and GC−MS equipment [22]. Although this study claims that the sulfur-volatile content in garlic was evaluated, a liquid extract was obtained and subsequently analyzed, instead of using a sampling device to obtain the volatile fraction.

The absence of in-depth studies on the volatile no sulfur compounds profile of garlic and on the behavior of volatile compounds in garlic when subjected to different heating times constituted the objectives of the present research, namely: (i) to optimize a cheap and easy analytical method for garlic volatiles based on HS–GC−MS without the need for an SPME step; (ii) to identify the compounds that conform the volatile profile of garlic, especially those which have so far not received enough attention; (iii) to differentiate the volatile profiles of three garlic varieties cultivated in the South of Spain; and (iv) to study differences in the influence of preset heating times on the volatile profile of each variety.

2. Experimental

2.1. Samples

Fresh garlic from three varieties, *Purple* (var. Rocambole), *White* (var. Porcelain) and *Chinese* (var. Turban), were provided by La Abuela Carmen (Montalbán, Spain). Four bulbs of each variety were stored at –20 ºC and, prior to analysis, the cloves were separated and chopped, located into the HS vial and encapsulated. The analysis was carried out immediately after pre-treatment to avoid enzymatic degradation of the sample. Each sample was analyzed in duplicate.

2.2. Apparatus

Ten-mL HS vials sealed by 20 mm aluminium vial caps (Análisis Vínicos, Tomelloso, Spain), with 20 mm silicone/PTFE septa (Análisis Vínicos) placed in the rack of a 7694E headspace autosampler from Agilent (Palo Alto, CA, USA) were used. An Agilent 7890B gas chromatograph coupled to an Agilent 5977A mass spectrometer was used for appropriate separation and detection of volatile compounds. A Factor VF-5ms fused silica capillary column (30 m × 0.25 mm I.D., 0.25 μm film thickness, Varian) completed the experimental set-up.

2.3. HS−GC−MS analysis

Vial, loop and transfer line temperatures were set at 103, 113, and 123 ºC, respectively. The vial was heated without shaking for 10 min and pressurized for 12 s at 30 psi; the loop filling and equilibration times were set at 12 and 3 s, respectively; and the injection time was set at 45 s. The sample was chopped and 2 g of it was placed in the HS vial. Three mL of the headspace content was injected into the chromatograph. The injector temperature was set at 180 ºC and injection was in an 1/5 split mode. The gas flow was set at 1 mL/min. The oven temperature was programmed as follows: initial temperature 40 ºC (held for 5 min) and increased at 10 ºC/min to 250 ºC (held for 5 min). The total analysis time took 31

min, and 3 min extra time was necessary for re-establishing and equilibrating the initial conditions. The single quadrupole mass spectrometer was operated in the scan mode, for which the instrumental parameters were set as follows: transfer line, source and quad temperatures were kept at 250, 230, and 180 ºC, respectively; electron energy was set at 70 eV, data acquisition was set between 30 *m/z* and 500 *m/z* and the solvent delay was programmed for 2 min.

2.4. Experimental design

Optimization of HS principal parameters was supported on a Box-Behnken design by application of response surface methodology. This design evaluated 3 levels for each studied factor: vial temperature (X_1), vial equilibration time (X_2) and injection time (X_3). The resulting experimental design included fifteen experiments by defining the minimum, maximum and average values of each parameter: 60, 90 and 120 ºC for vial temperature; 5, 7.5 and 10 min for vial equilibration time; and 12, 3 and 45 s for injection time. Seven of the most intense peaks in different sections of the chromatogram were selected as response variables $-Y_k$ (x). For the first peak, the minimum response was considered as optimal since this peak is generated by compounds of low molecular mass and high volatility, suspicious of being generated by degradation. The experimental data were fitted into a second order polynomial model by the following equation:

$$Y_i = \beta_0 + \beta_m X_m + \beta_n X_n + \beta_{mm} X_m^2 + \beta_{mn} X_m X_n + \beta_{nn} X_n^2$$

A desirability function approach was used to select the condition for each factor that resulted in the best concentration of volatiles in the HS according to the following equation:

$$D = (d_1(Y_1) d_2(Y_2) \dots d_n(Y_n))^{1/n}$$

being $d_i(Y_i)$ a desirability function for each response Y_i (x). The $d_i(Y_i)$ function is equal to 1 representing a completely desirable or, in this case, the ideal response value, obtained by the surface response model. The results were processed using

the software Statgraphics Centurion XVI (StatPoint Technologies 2011, USA).

2.5. Data processing and statistical analysis

Qualitative Analysis software (version 7.0, Agilent Technologies, Santa Clara, CA, USA) was used to process all data obtained by GC−MS. Treatment of the raw data files started by deconvolution of potential molecular features (MFs) by the algorithm included in the software, which considered all ions exceeding 2000 counts for the absolute area parameter. The NIST Mass Spectral Search Program v. 11.0 (NIST, Washington, DC, USA) was used for spectral search (Mainlib and Replib libraries). Tentative identification was supported on correlation between experimental and database spectra above 650 in normal search mode.

Peaks corresponding to identified entities were integrated in all samples and the resulting areas were compiled in a matrix using a *.csv* file. The matrix was exported to Mass Profiler Professional (MPP) software package (Version 12.0, Agilent Technologies, Santa Clara, CA, USA), where the data set was treated by Principal Component Analysis (PCA) to evaluate the behavior of the three garlic varieties under preset heating times.

3. Results and discussion

3.1. Optimization of the HS parameters

The optimization study was focused on the three main HS parameters that allow obtaining the highest concentration of volatile compounds in the headspace of the vial. As described under materials and methods, a response surface methodology was used for this purpose. Independent studies were carried out for each selected peak of the chromatogram (see Electronic Supplementary Material Figure S1), and the results were combined in a desirability response surface with capability to define the optimum conditions to obtain the maximum signal for peaks 2-to-7 (identified as diallyl sulfide, 1,3-dithiane, diallyl disulfide, allyl 1-

propenyl disulfide, 3-vinyl-1,2-dithiacyclohex-5-ene and diallyl trisulfide, respectively) and the minimum signal for peak 1 (the sum of acetone and 2-propen-1-ol). This criterium was applied to obtain the maximum signal from compounds of interest in garlic with the greatest variety of them, and also with the minimum signal from less important compounds produced by degradation of higher compounds. The resulting desirable conditions are listed in Table 1, while Fig. 1 shows the response surface reported by this study. As can be seen in the figure, the desirable temperature in the vial was 103 ^{o}C, slightly above the boiling point of water, which suggest that volatile compounds of garlic are stored in the aqueous intracellular medium. The injection time adopted a high value (0.75 min), without reaching the maximum studied value. Finally, the desirable value of vial equilibration time resulted 10 min, the maximum value studied. Therefore, the heating kinetics was studied to know the behavior of the target compounds at vial equilibration times longer than 10 min. Fig. 2 shows the evolution of the intensity of the target compounds to reach the equilibration time, where an overall decreased intensity of the target peaks after 60 min of equilibration time can be observed. Nevertheless, at 30 min of heating time compounds such as 3-dithiane, diallyl sulfide, 3-vinyl-1,2-dithiacyclohex-5-ene and diallyl trisulfide experienced a slight increase of intensity; increase that was more noticeable for the peak corresponding to the sum of acetone and 2-propen-1-ol. These last compounds are characterized by low molecular mass and high volatility, and they can be considered as final degradation products. Thus, a heating time of 10 min was considered the best to obtain the volatile profile of garlic at the working temperature.

Table 1. Optimization of the main HS parameters by a desirability study to maximize the concentration of volatile compounds in the HS vial.

Factor	Low	High	Optimum
Vial temperature (°C)	60.0	120.0	103
Vial equilibration time (min)	5.0	10.0	10.0
Injection time (s)	12	48	45

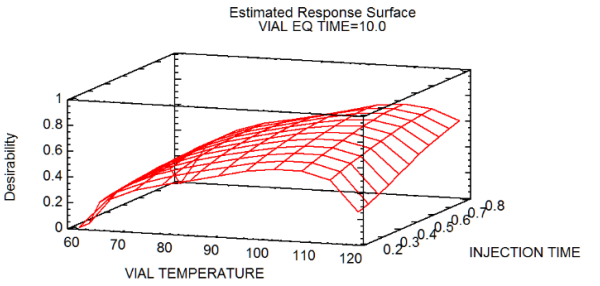

Figure 1. Response surface associated to the desirability conditions for the main HS parameters. Optimization was carried out monitoring 7 representative compounds by HS–GC–MS.

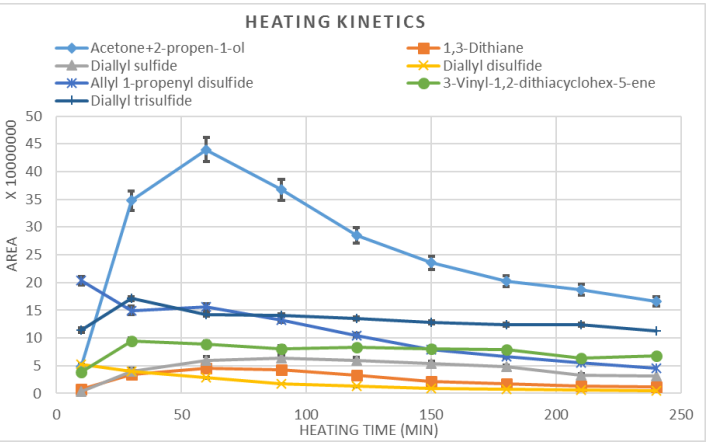

Figure 2. Heating kinetics of monitored compounds, expressed as peak area *versus* heating time.

3.2. Tentative identification of volatile compounds

The headspace of the vial containing the chopped garlic was generated under the desirability conditions and analyzed by GC−MS as described under Materials and methods. Tentative identification of the peaks was carried out by comparison of the resulting spectra with the theoretical spectra stored in the NIST database, establishing a cut on the match factor of 650 (65% correlation between the experimental and theoretical spectra). Also, an alkanes standard mixture was analyzed to calculate the experimental RT index to support identification by an alkanes calibration model. Probably due to the different matrices, gas in the case of the HS of the samples and liquid in the case of the alkanes standard mixture, a mismatch between the experimental and the theoretical RT index (obtained from the NIST) was produced. To overcome it, a correlation plot was generated to obtain a corrected RT index by extrapolation of the experimental value in the plot (see Electronic Supplementary Material Figure S2). Table 2 lists the tentatively identified compounds, the main parameters that support identification, and also stresses those identified for the first time. The tentatively identified compounds were classified into derivatives of S-alk(en)yl-L-cysteine, flavor compounds, and others. Flavor compounds were classified as a function of their flavor, as listed in http://www.thegoodscentscompany.com/.

Table 2 Volatiles identified in the headspace generated from garlic.

Compound	CAS	RT (min)	RT Index	Theoretical RT Index	Match	R. Match	Mass (Da)	Formula	Principal fragments
Derivatives from S-alk(en)yl-L-cysteine									
Dimethyl sulfide	75-18-3	1.67	553	455	813	815	58.0	C_4H_6O	32.0, 41.0, 43.0, 56.0
Allyl mercaptan	870-23-5	2.07	574	562	817	831	74.0	C_3H_6S	39.1, 41.1, 72.1, 74.0
Allyl methyl sulfide	10152-76-8	3.12	629	660	813	820	88.0	C_4H_8S	39.0, 45.0, 73.0, 88.0
Dimethyl disulfide	624-92-0	4.07	679	722	892	910	94.0	$C_2H_6S_2$	45.0, 55.0, 84.0, 93.9
Diallyl sulfide	592-88-1	7.42	854	849	820	881	114.1	$C_6H_{10}S$	39.0, 45.0, 73.0, 114.0
Allyl n-propyl sulfide	27817-67-0	7.81	875	859	828	867	116.1	$C_6H_{12}S$	32.0, 43.0, 44.0, 55.0
Allyl 1-propenyl sulfide	122156-02-9	8.18	894				114.1	$C_6H_{10}S$	32.0, 45.0, 99.0, 114.0
Di(1-propenyl) sulfide	65819-74-1	8.27	899	884	750	800	114.1	$C_6H_{10}S$	32.0, 41.0, 45.0, 99.0
1,3-Dithiane	505-23-7	8.85	912	1002	768	772	120.2	$C_4H_8S_2$	39.0, 41.0, 45.0, 119.9
Allyl methyl disulfide	2179-58-0	9.08	941	911	654	698	120.0	$C_4H_8S_2$	32.0, 41.0, 72.0, 115.9
Methyl 1-propenyl disulfide	10152-77-9	9.43	960	928	750	795	120.0	$C_4H_8S_2$	45.0, 72.0, 75.0, 119.9
Dimethyl trisulfide	3658-80-8	10.09	995	972	902	927	126.0	$C_2H_6S_3$	45.0, 63.9, 78.9, 125.9
Diallyl disulfide	2179-57-9	12.25	1108	1099	865	872	146.0	$C_6H_{10}S_2$	39.0, 41.1, 81.0, 146.0
Allyl 1-propenyl disulfide	122156-03-0	12.52	1122				146.0	$C_6H_{10}S_2$	41.1, 45.0, 73.0, 146.0
Di(1-propenyl) disulfide	53925-82-9	12.63	1127	1103	816	819	146.0	$C_6H_{10}S_2$	41.1, 45.0, 73.0, 146.0
Allyl methyl trisulfide	34135-85-8	13.28	1162	1161	656	715	152.0	$C_4H_8S_3$	41.0, 45.0, 73.0, 87.0
3-Vinyl-1,2-dithiacyclohex-4-ene	62488-52-2	14.10	1205	1134	778	926	144.0	$C_6H_8O_2$	79.0, 97.0, 111.0, 144.0
3-Vinyl-1,2-dithiacyclohex-5-ene	62488-53-3	14.48	1224	1155	900	956	144.0	$C_6H_8O_2$	71.0, 72.0, 111.0, 144.0
Benzothiazole*	95-16-9	14.66	1234	1208	863	925	135.0	C_7H_5NS	32.0, 44.0, 108.0, 134.9
Diallyl trisulfide	2050-87-5	15.72	1292	1350	854	875	178.0	$C_6H_{10}S_3$	39.0, 41.1, 73.0, 113.0
Di(1-propenyl) trisulfide	115321-81-8	16.15	1312				178.0	$C_6H_{10}S_3$	41.0, 45.0, 73.0, 114.0
Diallyl tetrasulfide	2444-49-7	18.95	1459	1510	812	826	210.0	$C_6H_{10}S_4$	39.0, 41.0, 73.0, 146.0

Cont. Table 2

Flavor compounds									
Green/Floral flavor									
2-Butenal	4170-30-3	2.53	598	615	943	946	70.0	C_4H_6O	39.0, 41.0, 69.0, 70.0
3-Penten-2-one*	625-33-2	3.98	674	714	799	927	84.1	C_5H_9O	41.0, 43.0, 69.0, 84.0
2-Methyl-4-pentenal	108-11-2	4.19	685	732	796	928	98.1	$C_6H_{10}O$	41.0, 43.0, 45.0, 55.0
2-Hexanol*	626-93-7	4.38	695	780	720	779	102.1	$C_6H_{14}O$	39.0, 55.0, 83.0, 84.0
5-Hexen-2-one*	109-49-9	5.01	728	744	908	909	98.1	$C_6H_{10}O$	39.0, 43.0, 55.0, 83.0
5-Hexenal*	764-59-0	5.35	746	796	875	918	98.1	$C_6H_{10}O$	39.0, 41.0, 54.0, 80.0
Hexanal*	66-25-1	5.74	767	803	890	892	100.1	$C_6H_{12}O$	41.0, 44.0, 56.1, 72.0
4-Heptenal	6728-31-0	7.09	837	870	840	842	112.1	$C_7H_{12}O$	41.0, 53.0, 67.0, 68.0
2,4-Hexadienal	142-83-6	8.77	925	877	833	902	96.1	C_6H_8O	53.0, 67.0, 96.0, 81.0
Benzaldehyde*	100-52-7	9.91	985	920	909	918	106.0	C_7H_6O	51.0, 77.0, 92.0, 106.0
3-Methyl-benzaldehyde*	620-23-5	12.05	1097	1060	884	897	120.1	C_8H_8O	65.0, 91.0, 119.0, 120.0
Roasted flavor									
3-Methyl butanal*	590-86-3	2.59	601	643	930	930	86.0	$C_5H_{10}O$	39.0, 41.0, 69.0, 70.0
2-Methyl butanal*	96-17-3	2.70	607	643	912	912	86.0	$C_5H_{10}O$	41.0, 43.0, 57.1, 58.0
2-Pentenal	1576-87-0	2.96	621	697	885	890	84.1	C_5H_8O	39.0, 41.0, 55.0, 83.0
Furfural*	98-01-1	6.72	818	831	960	961	96.0	$C_5H_4O_2$	39.0, 67.0, 95.0, 96.0
3-Ethyl pyridine*	536-78-7	9.86	982	937	879	914	107.1	C_7H_9N	51.0, 77.0, 105.0, 106.0
Sweet flavor									
1-Hydroxy-2-propanone*	116-09-6	3.72	660	698	832	873	74.0	$C_3H_6O_2$	31.0, 43.0, 45.0, 74.0
2-Acetylfuran*	1192-62-7	8.80	927	878	943	946	110.0	$C_6H_6O_2$	39.0, 42.0, 81.0, 108.0
Various flavor									
2-Ethyl-2-butenal (pungent)*	19780-25-7	6.30	796	791	935	938	98.1	$C_6H_{10}O$	41.0, 55.0, 69.0, 98.0
5-Ethyl-2-methyl-pyridine (nutty)*	104-90-5	11.21	1053	1000	845	882	121.1	$C_8H_{11}N$	45.0, 77.0, 106.0, 121.0
Other compounds									
Acetone*	67-64-1	1.67	569	455	813	816	58.0	C_3H_6O	40.0, 41.0, 43.0, 56.0
2-Propen-1-ol	107-18-6	1.86	579	552	945	947	58.0	C_3H_6O	31.1, 39.1, 57.0, 58.0
2-Methylene-4-pentenal	108-11-2	4.26	689	763	829	834	96.1	C_6H_8O	39.0, 41.0, 67.0, 95.0

The asterisk indicates compounds tentatively identified for the first time.

Twenty-one volatile organosulfur compounds were tentatively identified in the content of the headspace. These compounds are originated from S-alk(en)yl-L-cysteine by a series of chain reactions. This derivative of L-cysteine is oxidized, generating S-alk(en)yl-L-cysteine-S-oxides among which alliin is the most abundant in garlic. When garlic is chopped, the enzyme alliinase is activated and transforms alliin and the rest of alk(en)yl-L-cysteine-S-oxides into sulfenic acid intermediates. In the aqueous intracellular medium, these intermediates are rapidly condensed into thiosulfinates, being allicin, formed from alliin, the major thiosulfinate present in garlic. These compounds are very unstable and their subsequent reactions lead to a wide variety of sulfur derivatives, most of them volatile [13]. Within this group of volatiles, diallyl mono-, di and trisulfide and 1,3-dithiane are four of the most characteristic sulfur volatiles present in garlic. The sulfur volatiles presented a similar fragmentation pattern, as can be seen in Table 2, where the most intense fragments of each compound are listed. Nevertheless, isomers such as diallyl and di(1-propenyl) presented a distinguishable fragmentation pattern, as shows Figure 3; the di(1-propenyl) isomer presented a number of fragments higher than the diallyl isomer, while the former provided higher intensity of the major fragments. The spectrum of the diallyl isomer shows a major fragment at *m/z* 41.1, generated by rupture of the bond between the sulfur atom and the alpha carbon. This fragment was also present in the spectrum corresponding to di(1-propenyl) isomer but its relative intensity was lower than that of the diallyl isomer. The di(1-propenyl) isomer provided one other intense fragment at *m/z* 73.0 generated by rupture of the sulfur–sulfur bond; thus, the presence of a double bond between the alpha and beta carbons in the di(1-propenyl) isomer stabilizes the alpha carbon–sulfur bond. The spectra of both compounds were also characterized by the occurrence of characteristic fragments generated by cyclization. Two of these fragments, *m/z* 39.0 and 45.0, are produced by intramolecular fragmentation of both cycles. Additionally, the two cycles can be opened to generate the *m/z* 81.0 and 104.9 fragments from the diallyl isomer and

the di(1-propenyl) isomer, respectively. Concerning the allyl 1-propenyl isomer, Table 2 does not list theoretical RT Index, Match and R. Match since these compounds are not present in the NIST database; however, their spectra are characterized by the same fragments as diallyl and di(1-propenyl) isomers. Also, Radulović et al. identified these compounds and obtained for the three isomers the same elution order as in this study. The same applies to the identification of di(1-propenyl) trisulfide.

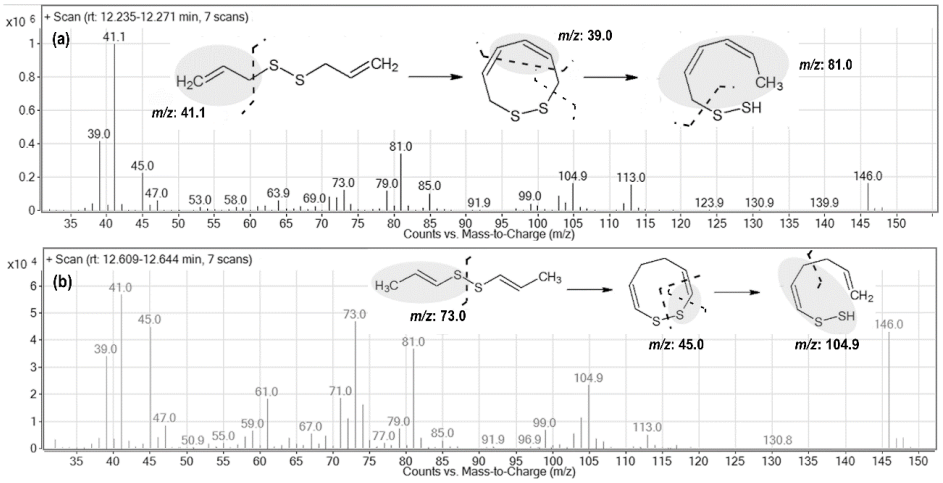

Figure 3. MS spectra and fragmentation patterns of diallyl disulfide (a) and di(1-propenyl) disulfide (b).

The structural stability caused by the presence of one double bond between the alpha and beta carbons can also be observed in the fragmentation of the 3-vinyl-dithiacylohex-4-ene and 3-vinyl-dithiacylohex-5-ene isomers (see Electronic Supplementary Material Figure S3). The first isomer, originated from the cyclization of the diallyl disulfide, is fragmented by rupture of the alpha carbon–sulfur and sulfur–sulfur bonds, generating the fragments *m/z* 79.0, 97.0 and 111.0. On the other hand, 3-vinyl-dithiacylohex-5-ene results from cyclization of allyl 1-propenyl disulfide, yielding as major fragment the *m/z* 72.0 generated by rupture of the heterocycle through the sulfur–sulfur bond. This fragmentation leads to two

fragments at *m/z* 72.0, which explain the high intensity of the peak in the spectra of 3-vinyl-dithiacylohex-5-ene.

Tentatively identified compounds that provide flavor to garlic constitute the second group of relevant volatile components. Eleven compounds with green or floral flavors were identified in the headspace from garlic samples, which also included five hexane derivatives. Five compounds with roasted flavor were also identified, among which furfural (which presents a roasted almond flavor) is the most remarkable. This compound, characteristic of the Maillard reaction, results from the degradation of a pentose sugar; as does a derivative from furfural, 2-acetylfuran, also identified in the headspace, which provides a characteristic sweet flavor, quite similar to 1-hydroxy-2-propanone. Finally, two compounds with different flavor were tentatively identified in the headspace: 2-ethyl-2-butenal, with a pungent flavor, and 5-ethyl-2-methyl-pyridine, characterized by a nutty flavor.

The last group encompasses three compounds without flavor characteristics (acetone, 2-methylene-4-pentenal and 2-propen-1-ol), being the former tentatively identified for the first time in the headspace from garlic, all of them with a high volatility as their peaks appeared in the chromatogram within the first four min.

3.3. Comparison of garlic varieties

Purple, *White* and *Chinese* garlic varieties were analyzed by the proposed HS–GC−MS method. Table 3 shows the percentage referred to as the total area of the tentatively identified compounds in the three varieties. Within the organosulfur compounds, it is worthy distinguishing that diallyl disulfide and diallyl trisulfide are major garlic volatiles which represented a relative concentration of 51.72% for *Chinese* garlic, 43.37% for *White* garlic and 42.32% for *Purple* garlic. Also, *Chinese* garlic was the variety with a higher number of sulfur volatiles, being allyl methyl disulfide the unique no detected volatile in this sample, and only detected in *White*

garlic. On the other hand, benzothiazole and di(1-propenyl) trisulfide were only detected in *Chinese* garlic.

Concerning green or floral flavor compounds, *White* garlic must be highlighted since it provided the major variety of these volatiles; however, *Purple* garlic presented the highest concentration of 2-butenal, the major compound of this group in all the samples. Comparing the total concentration of these volatiles (11.87% for *Chinese* garlic, 14.38% for *White* garlic and 15.01% for *Purple* garlic) it can be said that the *Purple* variety provided a major green and floral flavor. Roasted and sweet flavor compounds were not detected or detected at a concentration below 1%. Only 3-methyl butanal was detected in the three varieties, while 2-mehtyl butanal was only detected in *White* garlic, and 2-pentenal was detected in *Chinese* and *White* garlic, but at a concentration below 0.1%. Finally, 2-ethyl-2-butenal, with a pungent flavor, was detected in the three varieties, but at a high concentration in *White* and *Purple* garlics.

Acetone, 2-propen-1-ol, and 2-methylene-4-pentenal were also identified in the varieties under study, with a different relative concentration depending on the variety. Acetone and 2-porpen-1-ol are clearly the major compounds within this group, the sum of both representing more than 5% in all cases. It should be mentioned that the concentration of acetone was higher in *White* garlic, while 2-propen-1-ol was more concentrated in *Purple* garlic.

Table 3. Relative concentration (percentage of the total area) of the compounds identified in the three garlic varieties: *Purple*, *Chinese* and *White* garlic.

Compound	Garlic varieties		
	Chinese	*White*	*Purple*
Compounds derived from S-alk(en)yl-L-cysteine			
Dimethyl sulfide	0.20	0.35	0.06
Allyl mercaptan	0.14	–	1.4
Allyl methyl sulfide	1.1	2.3	3.6
Dimethyl disulfide	1.7	3.1	1.2
Diallyl sulfide	3.5	6.7	4.8
Allyl n-propyl sulfide	0.05	0.06	–
Allyl 1-propenyl sulfide	0.08	0.11	0.06
Di(1-propenyl) sulfide	0.12	0.29	0.16
1,3-Dithiane	3.0	2.9	5.4

Allyl methyl disulfide	–	0.04	–
Methyl 1-propenyl disulfide	0.33	0.33	0.33
Dimethyl trisulfide	0.13	0.18	0.74
Diallyl disulfide	26.4	24.8	21.0
Allyl 1-propenyl disulfide	2.8	1.7	0.90
Di(1-propenyl) disulfide	7.3	5.0	2.6
Allyl methyl trisulfide	5.0	4.6	8.8
3-Vinyl-1,2-dithiacyclohex-4-ene	0.38	0.19	0.26
3-Vinyl-1,2-dithiacyclohex-5-ene	1.1	0.55	0.92
Benzothiazole	0.05	–	–
Diallyl trisulfide	25.3	18.5	21.4
Di(1-propenyl) trisulfide	0.38	–	–
Diallyl tetrasulfide	0.64	0.32	0.21
Flavor compounds			
Green/Floral flavor			
2-Butenal	7.8	11.0	12.2
3-Penten-2-one	0.05	0.08	0.04
2-Methyl-4-pentenal	0.24	0.40	0.25
2-Hexanol	–	0.13	–
5-Hexen-2-one	0.32	0.24	0.18
5-Hexenal	0.48	0.55	0.29
Hexanal	0.47	0.25	0.28
4-Heptenal	1.8	1.5	1.0
2,4-Hexadienal	0.64	0.23	0.74
Benzaldehyde	0.03	0.04	–
3-Methyl-benzaldehyde	–	–	–
Roasted flavor			
3-Methyl butanal	0.29	0.53	0.48
2-Methyl butanal	–	0.16	–
2-Pentenal	0.06	0.08	–
Furfural	–	–	–
3-Ethyl pyridine	–	–	–
Sweet flavor			
1-Hydroxy-2-propanone	–	–	–
2-Acetylfuran	–	–	–
Other flavors			
2-Ethyl-2-butenal (pungent)	0.81	1.4	1.9
5-Ethyl-2-methyl-pyridine (nutty)	–	–	–
Other compounds			
Acetone	1.4	4.1	1.9
2-Propen-1-ol	4.5	4.9	5.3
2-Methylene-4-pentenal	0.38	0.33	0.29

The hyphen substitutes the compounds that were not detected in the sample.

3.4. Heating kinetics of the three garlic varieties

The pool of each sample was divided into eight 2-g portions, each of them placed in a headspace vial and subjected to 103 ^{o}C for preset times (10, 20, 30, 40, 50, 60, 90 or 120 min), then analyzed by HS–GC−MS as described under Materials and methods. Peaks corresponding to tentatively identified compounds were

integrated and the resulting area was used to quantify the compounds as a relative percentage of the total sum of the peak areas. The results from the heating kinetics for each type of sample are shown in Electronic Supplementary Material Table S1. As can be seen, the three garlic varieties are rich in sulfur volatiles, the sum of which is more than 50% of the total peak area for all the studied heating times. It should be emphasized that diallyl disulfide and diallyl trisulfide are major compounds in the samples, reaching percentages above 18% in all instances. Literature on garlic shows that diallyl mono-, di, tri- and tetrasulfide are four of the most common volatiles generated from S-alk(en)yl-L-cysteine; therefore, their evolution with the heating time and temperature is the key of the presence and concentration of sulfur volatiles [13]. Fig. 4 shows a plot of percentage *versus* heating time for each of the allyl sulfides discussed above. The three varieties showed a similar behavior: while diallyl sulfide and diallyl tetrasulfide increased their relative concentration in the headspace with the heating time, diallyl disulfide and diallyl trisulfide significantly decreased during the same interval, even to one half of their initial concentration in some cases.

Concerning tentatively identified compounds contributing to flavor, the headspace from the three varieties was rich in compounds with green or floral flavor, while sweet flavor compounds were never detected in *Chinese* garlic and no detected during the first 20 min of heating in *Purple* and *White* garlic. 2-Butenal was the major green flavor compound detected in all garlic samples; however, its concentration drastically decreased within 10–20 min heating, probably by degradation. Within the group of roasted flavor, furfural was the most significant compound, detected in the three varieties of samples after 20 min of heating, which reveals activation of Maillard reactions in garlic when heated for more than 10 min. 2-Acetylfuran and 5-methyl furfural, compounds with a sweet flavor, are also products of the Maillard reaction, as derivatives from furfural. However, they were only detected within the 50–60 min of heating in *White* garlic. Concerning the rest of compounds with various flavors, only two compounds were relatively

quantified: 2-ethyl-2-butenal, detected in the three varieties, and with concentration gradually decreased during heating in all instances (thus, it can be assumed that the pungent flavor provided by this compound is characteristic of fresh garlic); and 5-ethyl-2-methyl-pyridine, only detected in *Purple* garlic after 20 min heating and with progressive decreased concentration by increasing the heating time.

Finally, the remaining compounds that do not provide flavor were relatively quantified in all varieties, where it is remarkable the behavior of acetone, which strongly increased its concentration after 10–20 min heating (more than 10% of the total peak area).

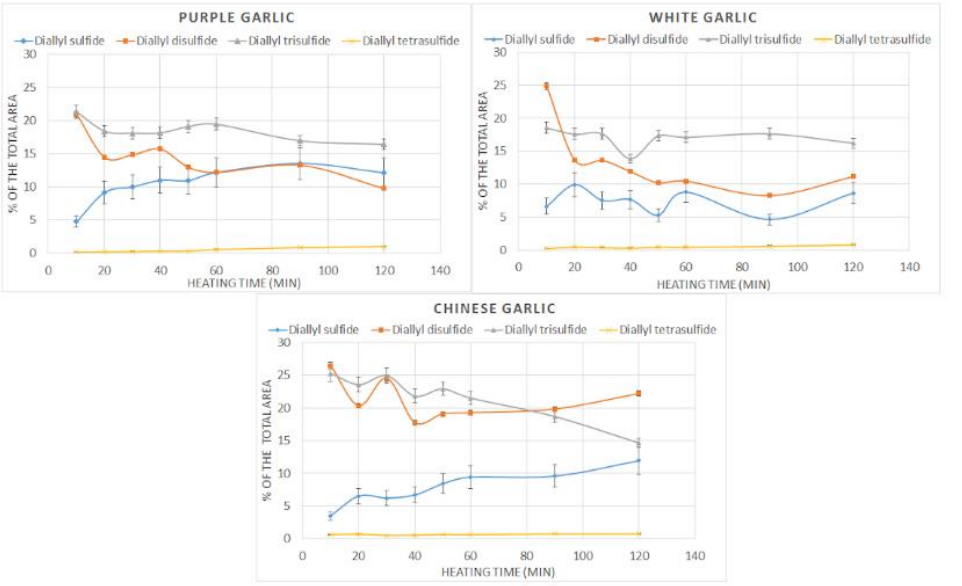

Figure 4. Evolution of the relative concentration (percentage of the total peak area) of diallyl sulfide, diallyl disulfide, diallyl trisulfide and diallyl tetrasulfide in the three varieties during the heating time.

A matrix was created from the obtained data as described in the Material and methods section and treated by PCA to evaluate differences among samples and contribution of each compound. Fig. 5 shows the PCA plot of heating kinetics of

the three garlic varieties: *Purple* (P), *Chinese* (C) and *White* (W) garlic. Component 3, plotted in the *z*-axis, clearly differentiates between garlic varieties, while Components 1 and 2, plotted in the *x* and *y*-axis, respectively, mark the evolution of the samples according to the heating time. The compounds with major contribution to Component 3 were dimethyl sulfide, acetone and 3-penten-2-one; thus, the concentration of these compounds on the HS justifies differentiation of *White* garlic regarding to the rest. On the opposite side, allyl methyl sulfide, dimethyl trisulfide and methyl 2-propenyl trisulfide presented the largest negative contribution to this Component, being responsible for the separation of *Purple* garlic samples in the PCA. Concerning to separation along the heating time, compounds with high values in the *x*-axis and low values in the *y*-axis are responsible for differentiation of garlic samples heated for 10 min. 2-Butenal, allyl mercaptan and benzothiazole provided the higher value on Component 1, while hexanal, 3-vinyl-1,2-dithiacyclohex-4-ene and 2-butenal were the compounds with the lowest values on Component 2. The presence of 2-butenal among the most significant compounds is consistent in this case with its behavior discussed above; thus, the concentration of this compound in the HS, indicative of garlic freshness, corresponds to low degradation at short heating times. On the other hand, compounds with a low value on Component 1 and a high value on Component 2 justify differentiation of samples with longer heating times. Diallyl tetrasulfide, 1-propenyl allyl sulfide and 2-methyl-4-pentenal presented the lowest value on Component 1, while 2-propen-1-ol, dimethyl trisulfide and 2-pentenal provided the highest value on Component 2. Although the identified volatile compounds classified as others are not characteristic of garlic and do not provide any flavor, they seem to be important for differentiation of both garlic varieties and fresh–heated garlic, since most of them are responsible for the variance along the three components.

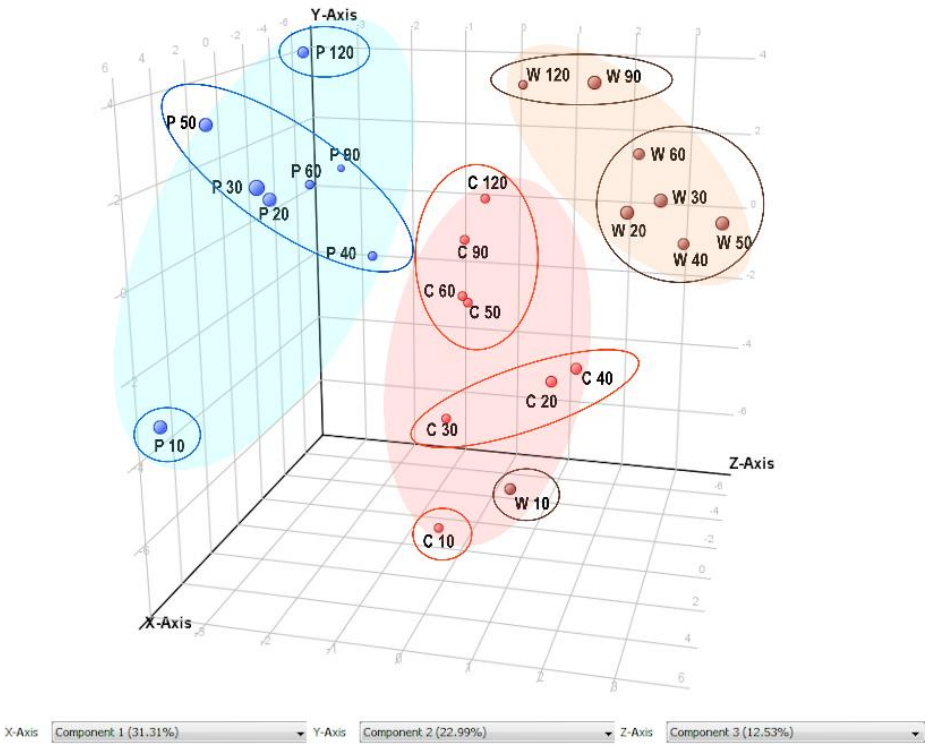

Figure 5. PCA of *Purple* (P), *Chinese* (C) and *White* (W) garlic samples heated for different times (numbers express min of heating).

4. Conclusions

An HS–GC−MS method is proposed for identification of the volatile profile of garlic. The proposed method was optimized to obtain the higher number of volatile compounds. The optimized method was applied to garlic samples, which led to tentative identification of 45 volatile compounds (17 of which were identified for the first time in garlic) by study of the fragmentation pattern of the most important sulfur compounds. The volatile profile of three garlic varieties — *Chinese*, *White* and *Purple*— was compared, showing that *Chinese* garlic is the richest in sulfur volatiles, and *Purple* garlic has the highest concentration of flavor

volatiles. The proposed method was also used to study the behavior of the three garlic varieties under different heating times. The three varieties presented a similar behavior, being flavor compounds the most illustrative of the evolution of garlic with the heating time. Green and floral volatiles, freshness indicators in garlic, drastically decrease their concentration, while roasted and sweet flavor compounds appear or increase their concentration by increasing the heating time. On the other hand, sulfur volatiles presented both trends depending on the compound. Finally, these results allowed PCA differentiation among both the varieties and the heating times, where it can be seen that all varieties had a similar trend when subjected to preset heating times, although they can be clearly distinguished among them.

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Supplementary information

Supplementary Table S1. Relative concentration (as percentage of the total area) of tentatively identified compounds in the samples heated for different times.

Compound	*White* garlic							
	10 min	20 min	30 min	40 min	50 min	60 min	90 min	120 min
Derivatives from S-alk(en)yl-L-cysteine								
Dimethyl sulfide	0.35	0.59	0.44	0.57	0.93	0.51	0.30	0.29
Allyl mercaptan	–	–	–	–	–	–	–	–
Allyl methyl sulfide	2.3	2.5	2.1	2.8	2.3	2.7	1.8	3.6
Dimethyl disulfide	3.1	2.2	2.0	2.2	2.1	1.6	1.4	1.4
Diallyl sulfide	6.7	9.9	7.5	7.7	5.3	8.8	4.6	8.7
Allyl n-propyl sulfide	0.06	0.12	0.10	0.07	0.06	0.10	0.05	0.07
Allyl 1-propenyl sulfide	0.11	0.17	0.10	0.12	0.08	0.11	0.06	0.12
Di(1-propenyl) sulfide	0.29	0.22	0.09	0.17	0.09	0.11	0.06	0.07
1,3-Dithiane	2.9	1.2	1.3	2.0	1.5	1.1	1.2	2.7
Allyl methyl disulfide	0.04	0.18	0.13	0.15	0.24	0.15	0.21	0.23
Methyl 1-propenyl disulfide	0.33	0.36	0.29	0.31	0.38	0.40	0.39	0.62
Dimethyl trisulfide	0.18	0.20	0.28	0.25	0.52	0.33	0.56	0.93
Diallyl disulfide	24.8	13.7	13.6	11.9	10.2	10.5	8.3	11.2
Allyl 1-propenyl disulfide	1.7	1.2	0.99	0.83	0.87	0.88	0.71	1.2
Di(1-propenyl) disulfide	5.0	3.9	2.9	2.6	2.8	2.9	2.5	3.0
Allyl methyl trisulfide	4.6	4.3	5.2	4.5	6.9	5.9	7.2	8.5
3-Vinyl-1,2-dithiacyclohex-4-ene	0.19	0.10	0.09	0.12	0.08	0.10	0.07	0.06
3-Vinyl-1,2-dithiacyclohex-5-ene	0.55	0.30	0.31	0.36	0.24	0.31	0.22	0.17
Benzothiazole	–	–	–	–	0.16	0.03	0.04	0.04
Diallyl trisulfide	18.5	17.6	17.6	13.9	17.4	17.1	17.75	16.2
Di(1-propenyl) trisulfide	–	–	–	–	–	–	–	–
Diallyl tetrasulfide	0.32	0.51	0.44	0.37	0.50	0.50	0.65	0.84
Flavor compounds								
Green/Floral flavor								
2-Butenal	11.0	0.55	0.62	1.7	0.61	0.32	0.19	0.09
3-Penten-2-one	0.08	0.18	0.14	0.27	0.22	0.15	0.07	0.09
2-Methyl-4-pentenal	0.40	1.0	0.62	0.75	0.70	0.95	0.74	0.99
2-Hexanol	0.13	–	–	–	–	–	–	–
5-Hexen-2-one	0.24	0.15	0.15	0.16	0.21	0.12	0.10	0.13
5-Hexenal	0.55	2.4	2.0	3.1	2.1	2.5	1.8	1.8
Hexanal	0.25	0.14	0.11	0.15	0.28	0.09	0.04	0.07
4-Heptenal	1.5	7.4	6.9	8.5	6.1	8.3	6.9	7.2

2,4-Hexadienal	0.23	0.06	–	–	–	–	–	–
Benzaldehyde	0.04	0.15	0.09	0.16	0.10	0.11	0.07	0.14
3-Methyl-benzaldehyde	–	0.11	0.12	0.10	0.09	0.13	0.16	0.09
Roasted flavor								
3-Methyl butanal	0.53	0.03	0.03	0.14	0.08	0.05	0.03	0.12
2-Methyl butanal	0.16	0.05	0.03	0.09	0.10	0.08	0.10	0.11
2-Pentenal	0.08	0.27	0.27	0.25	0.23	0.29	0.27	0.20
Furfural	–	–	0.08	0.09	0.17	0.14	0.24	0.17
3-Ethyl pyridine	–	–	–	–	–	–	–	–
Sweet flavor								
1-Hydroxy-2-propanone	–	–	–	0.06	0.13	0.07	0.14	0.06
2-Acetylfuran	–	–	–	0.07	0.07	0.04	–	–
Other flavors								
2-Ethyl-2-butenal (pungent)	1.4	0.81	0.44	0.49	0.65	0.34	0.30	0.22
5-Ethyl-2-methyl-pyridine (nutty)	–	–	–	–	–	–	–	–
Other compounds								
Acetone	4.1	5.7	8.4	10.3	9.6	7.1	6.2	5.2
2-Propen-1-ol	4.9	20.8	23.4	21.0	24.8	24.2	34.4	23.1
2-Methylene-4-pentenal	0.33	0.53	0.45	0.43	0.46	0.32	0.10	0.05

The hyphen substitutes the compounds that were not detected in the sample.

Compound	***Chinese* garlic**							
	10 min	**20 min**	**30 min**	**40 min**	**50 min**	**60 min**	**90 min**	**120 min**
Compounds derived from S-alk(en)yl-L-cysteine								
Dimethyl sulfide	0.20	0.22	0.19	0.26	0.19	0.17	0.18	0.14
Allyl mercaptan	0.14	0.12	0.11	0.06	0.08	0.09	0.15	0.48
Allyl methyl sulfide	1.1	1.5	1.7	1.1	2.1	2.1	1.7	1.6
Dimethyl disulfide	1.7	1.3	1.1	1.4	1.1	0.97	0.86	0.99
Diallyl sulfide	3.5	6.5	6.2	6.7	8.4	9.4	9.6	12.0
Allyl n-propyl sulfide	0.05	0.04	0.03	0.03	0.03	0.02	0.02	0.02
Allyl 1-propenyl sulfide	0.08	0.10	0.10	0.11	0.13	0.11	0.10	0.08
Di(1-propenyl) sulfide	0.12	0.08	0.11	0.07	0.09	0.07	0.06	0.03
1,3-Dithiane	3.0	1.6	2.4	1.5	1.9	1.6	2.1	3.7
Allyl methyl disulfide	–	0.05	0.04	0.06	0.06	0.05	0.07	0.08
Methyl 1-propenyl disulfide	0.33	0.20	0.25	0.23	0.29	0.22	0.22	0.12
Dimethyl trisulfide	0.13	0.10	0.14	0.14	0.18	0.14	0.17	0.16
Diallyl disulfide	26.4	20.3	24.5	17.8	19.1	19.3	19.8	22.2
Allyl 1-propenyl disulfide	2.8	1.8	2.2	1.7	1.9	1.6	1.5	0.88
Di(1-propenyl) disulfide	7.3	5.3	6.5	4.7	5.4	4.7	4.2	2.0

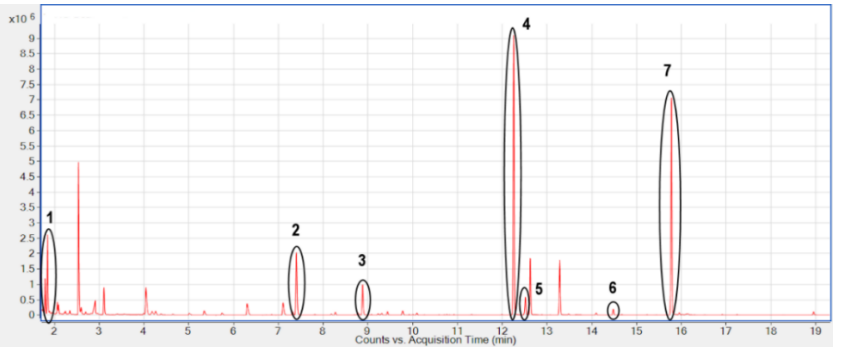
Allyl methyl trisulfide	5.0	4.0	4.9	4.5	5.2	4.4	4.4	3.9
3-Vinyl-1,2-dithiacyclohex-4-ene	0.38	0.37	0.34	0.31	0.28	0.29	0.26	0.15
3-Vinyl-1,2-dithiacyclohex-5-ene	1.1	0.94	0.89	0.92	0.69	0.66	0.57	0.34
Benzothiazole	0.05	0.02	0.03	–	–	–	–	–
Diallyl trisulfide	25.3	23.6	24.9	21.8	22.9	21.5	18.7	14.6
Di(1-propenyl) trisulfide	0.38	0.15	0.18	0.11	0.14	0.12	0.16	0.15
Diallyl tetrasulfide	0.64	0.72	0.50	0.53	0.65	0.65	0.74	0.74
Flavor compounds								
Green/Floral flavor								
2-Butenal	7.8	1.9	2.1	1.7	0.94	0.75	0.36	0.20
3-Penten-2-one	0.05	0.15	0.06	0.13	0.08	0.09	0.10	0.09
2-Methyl-4-pentenal	0.24	0.63	0.56	0.74	0.88	1.0	1.1	1.1
2-Hexanol	–	–	–	–	–	–	–	–
5-Hexen-2-one	0.32	0.22	0.18	0.26	0.16	0.12	0.09	0.21
5-Hexenal	0.48	1.3	0.87	1.5	1.4	1.3	1.3	1.2
Hexanal	0.47	0.22	0.23	0.28	0.21	0.15	0.14	0.13
4-Heptenal	1.8	6.4	4.2	6.8	7.0	7.5	8.3	8.0
2,4-Hexadienal	0.64	0.35	0.53	0.34	0.17	0.14	0.05	0.04
Benzaldehyde	0.03	0.09	0.07	0.13	0.09	0.10	0.10	0.10
3-Methyl-benzaldehyde	–	0.06	0.04	0.07	0.08	0.08	0.09	0.09
Roasted flavor								
3-Methyl butanal	0.29	0.05	0.06	0.06	0.04	0.04	0.05	0.05
2-Methyl butanal	–	–	–	–	0.03	0.03	0.04	0.04
2-Pentenal	0.06	0.14	0.11	0.08	0.19	0.08	0.08	0.08
Furfural	–	0.06	0.04	0.11	0.07	0.09	0.12	0.10
3-Ethyl pyridine	–	–	–	–	–	–	–	–
Sweet flavor								
1-Hydroxy-2-propanone	–	–	–	–	–	–	–	–
2-Acetylfuran	–	–	–	–	–	–	–	–
Other flavors								
2-Ethyl-2-butenal (pungent)	0.81	0.56	0.52	0.50	0.45	0.38	0.29	0.24
5-Ethyl-2-methyl-pyridine (nutty)	–	–	–	–	–	–	–	–
Other compounds								
Acetone	1.4	2.5	1.3	2.3	2.1	1.9	2.3	2.7
2-Propen-1-ol	4.5	15.5	10.8	20.2	14.6	17.6	19.6	21.1
2-Methylene-4-pentenal	0.38	0.38	0.55	0.43	0.38	0.38	0.16	0.08

The hyphen substitutes the compounds that were not detected in the sample.

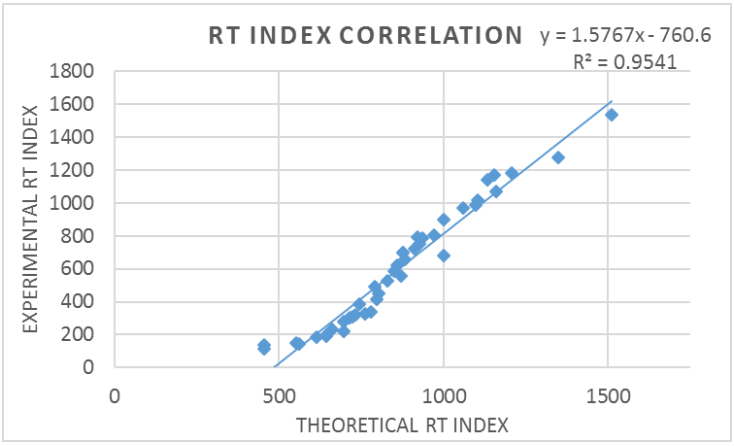
Compound	*Purple* garlic							
	10 min	20 min	30 min	40 min	50 min	60 min	90 min	120 min
Derivatives from S-alk(en)yl-L-cysteine								
Dimethyl sulfide	0.06	0.09	0.13	0.18	0.09	0.08	0.14	0.08
Allyl mercaptan	1.4	0.98	0.56	0.18	0.22	0.13	0.11	0.17
Allyl methyl sulfide	3.6	9.0	6.4	5.8	8.2	6.4	6.2	5.4
Dimethyl disulfide	1.2	1.2	1.2	1.5	1.1	1.3	1.5	1.4
Diallyl sulfide	4.8	9.2	10.0	11.0	11.0	12.2	13.6	12.1
Allyl n-propyl sulfide	–	–	–	–	–	–	–	–
Allyl 1-propenyl sulfide	0.06	0.13	0.11	0.14	0.12	0.15	0.13	0.11
Di(1-propenyl) sulfide	0.16	0.16	0.12	0.15	0.11	0.13	0.08	0.05
1,3-Dithiane	5.4	2.2	2.1	2.7	1.7	0.46	2.6	2.2
Allyl methyl disulfide	–	0.75	0.07	0.08	0.06	0.01	0.13	0.20
Methyl 1-propenyl disulfide	0.33	0.37	0.38	0.51	0.40	0.46	0.51	0.49
Dimethyl trisulfide	0.74	0.65	0.63	0.87	0.70	0.90	1.2	1.4
Diallyl disulfide	21.0	14.5	14.9	15.7	13.0	12.2	13.3	9.8
Allyl 1-propenyl disulfide	0.90	0.78	0.69	0.94	0.66	0.76	0.81	0.61
Di(1-propenyl) disulfide	2.6	2.5	2.2	2.8	2.2	2.3	2.3	1.7
Allyl methyl trisulfide	8.8	7.6	7.7	8.7	8.4	9.4	8.9	10.1
3-Vinyl-1,2-dithiacyclohex-4-ene	0.26	0.15	0.20	0.16	0.15	0.16	0.21	0.13
3-Vinyl-1,2-dithiacyclohex-5-ene	0.92	0.53	0.74	0.49	0.56	0.52	0.61	0.40
Benzothiazole	–	–	–	–	–	0.07	–	–
Diallyl trisulfide	21.4	18.4	18.1	18.2	19.1	19.5	17.0	16.4
Di(1-propenyl) trisulfide	–	–	–	–	–	–	–	–
Diallyl tetrasulfide	0.21	0.25	0.28	0.33	0.35	0.57	0.84	1.01
Flavor compounds								
Green/Floral flavor								
2-Butenal	12.2	1.4	0.84	1.9	1.0	1.2	0.67	0.19
3-Ppenten-2-one	0.04	0.07	0.06	0.12	0.04	0.11	0.13	0.05
2-Methyl-4-pentenal	0.25	0.54	0.59	0.70	0.69	0.83	1.0	0.94

2-Hexanol	–	–	–	–	0.08	0.04	0.04	0.10
5-Hexen-2-one	0.18	0.06	0.06	0.09	0.05	0.12	0.08	0.08
5-Hexenal	0.29	1.2	1.1	1.5	1.0	1.4	1.28	0.89
Hexanal	0.28	0.09	0.06	0.19	0.05	0.09	0.10	0.04
4-Heptenal	1.0	4.1	3.8	5.4	3.8	6.3	6.49	5.44
2,4-Hexadienal	0.74	0.09	0.12	0.11	0.05	0.05	–	–
Benzaldehyde	–	0.11	0.25	0.14	0.19	0.19	0.19	0.18
3-Methyl-benzaldehyde	–	0.12	0.15	0.10	0.15	0.18	0.15	0.19
Roasted flavor								
3-Methyl butanal	0.48	0.06	0.07	0.09	0.05	0.06	0.06	0.04
2-Methyl butanal	–	–	–	0.03	0.03	0.03	0.04	–
2-Pentenal	–	0.22	0.20	0.20	0.25	0.26	0.19	0.18
Furfural	–	0.08	0.08	0.05	0.11	0.08	0.08	0.12
3-Ethyl pyridine	–	–	–	–	–	0.07	0.14	0.13
Sweet flavor								
1-Hydroxy-2-propanone	–	–	–	–	–	0.06	0.05	0.23
2-Acetylfuran	–	–	–	–	–	–	–	–
Other flavors								
2-Ethyl-2-butenal (pungent)	1.9	0.59	0.76	1.0	0.50	0.88	0.57	0.26
5-Ethyl-2-methyl-pyridine (nutty)	–	0.05	0.14	0.09	0.07	0.13	0.20	0.23
Other compounds								
Acetone	1.9	2.7	2.8	3.1	2.0	2.3	2.3	2.0
2-Propen-1-ol	5.3	18.5	21.7	13.5	21.2	16.9	15.8	24.8
2-Methylene-4-pentenal	0.29	0.49	0.51	0.42	0.45	0.48	0.21	0.08

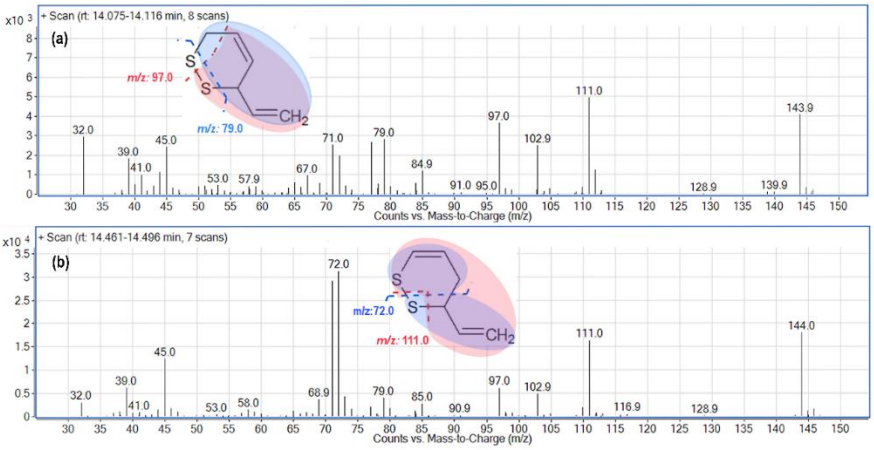
The hyphen substitutes the compounds that were not detected in the sample.



Supplementary Fig. S1. Peaks selected in the chromatogram for optimization of the main HS parameters.



Supplementary Fig. S2. Correlation plot between the theoretical RT index (obtained from the NIST) and the experimental RT index (calculated from an alkanes calibration model).



Supplementary Fig. S3. MS spectra and fragmentation patterns of 3-vinyl-dithiacylohex-4-ene (a) and 3-vinyl-dithiacylohex-5-ene (b).

CAPÍTULO VIII

Headspace–GC–MS volatile profile of black garlic *vs* fresh garlic: evolution along fermentation and behavior under heating

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Headspace–GC–MS volatile profile of black garlic *vs* fresh garlic: evolution along fermentation and behavior under heating

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Headspace–GC–MS volatile profile of black garlic *vs* fresh garlic: evolution along fermentation and behavior under heating

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ABSTRACT

Most healthy properties of fresh and black (a fermented derivative) garlic are attributed to volatile organosulfur compounds. In this research, the volatile profile of black garlic (from purple garlic) and its evolution along the fermentation process were evaluated using a headspace (HS) device coupled to a gas chromatography–mass spectrometry (GC–MS) arrangement. The volatile profiles of black and fresh garlic were compared by testing different heating times in the HS device. The HS–GC–MS analysis allowed identifying 51 volatile compounds that were classified into S-alk(en)yl-L-cysteine derivatives, flavor compounds and others. Significant changes in the volatile profile along fermentation allowed PCA differentiation among the process steps under study. Black and fresh garlic provided different volatile profiles, especially in flavor compounds, and presented different behaviors under heating. Especially remarkable was the evolution of sulfur volatiles.

Keywords Black garlic; HS−GC−MS; Sulfur volatiles; Flavor; Fermentation process.

1. Introduction

Garlic (*Allium sativum*) is widely used in cuisines around the world as seasoning ingredient, mainly in Asia, Africa and Europe. Historically, garlic was used by the Egyptians in several therapeutic formulas (Block, 1985), and by Greeks and Romans as healing agent. Expansion of garlic use to the whole Mediterranean basin (Griffiths, Trueman, Crowther, Thomas & Smith, 2002) led Spain to assume a historical heritage that converted this country in one of the major producers of garlic of the European Union, with a production of 173 million of tones of garlic in 2013 (FAOSTATS, 2013). Nowadays, a garlic derivative that is gaining popularity is fermented garlic, also known as black garlic due to its darkness color. Black garlic production bases on thermal processing of fresh garlic for 1–3 months at 60–80 ^{o}C under controlled humidity conditions, and without additives. Under these conditions a number of chemical reactions, such as Maillard and enzymatic reactions, change garlic color from white to black and drastically transform its flavor (Kim, Nam, Rico & Kang, 2012).

The first studies on garlic, carried out by Louis Pasteur (1858), attributed anti-bacterial properties to garlic. More recent studies have reported that garlic extracts act as anti-oxidant (Lee, Kim, Jung, Kim & Jang, 2005), anti-microbial (Whitmore & Naidu, 2000) and anti-asthmatic (Dorsch & Wagner, 1992) agents. Nowadays, attention focuses on cancer preventive properties of garlic (Block, 1993; Kamel, 2000; Miron, Mironchick, Mirelman, Wilchek & Rabinkov, 2003), as is the case with the study of Buiatti et al., (1989), who found a relationship between the decrease of gastric cancer risk and increased intake of garlic. This beneficial effect was related to the capability of garlic to reduce the concentration of nitrite in the gastric tract, as showed by Xing et al. (1982). Recent studies on black garlic have demonstrated that the traditional healthy properties of fresh garlic even rise in black garlic: the radical scavenging capability and total phenolic content were greater in black than in fresh garlic; thus, anti-oxidative properties are enhanced

by the fermentation process [Kim, Nam, Rico & Kang, 2012; Sato, Kohno, Hamano & Niwano, 2006, Kim, Kang & Gweon, 2013]. Also the intake of black garlic is related to protection against diabetes and obesity (Jung et al., 2011) and against UVB photodamage of the skin (Kim et al., 2012).

Most of the beneficial properties ascribed to fresh and black garlic have been related to the presence of organosulfur compounds (that also exist in the rest of the *Allium* family), and whose main components are thiosulfinates and sulfur volatiles. The former family —S-alk(en)yl-L-cysteine derivatives— seems to be responsible for many of the beneficial properties of garlic, despite they are unstable compounds, subjected to chain reactions that transform them into volatile sulfur compounds (Lanzotti, 2006). In addition to be responsible for the characteristic aroma of garlic, components of this fraction such as diallyl disulfide and diallyl trisulfide have demonstrated anti-cancer activity (Yang et al., 2006; Seki et al., 2008).

The methods so far proposed to determine the volatile profile of garlic require sample preparation prior to gas chromatography–mass spectrometry (GC–MS) analysis, which has been mainly based on solid phase microextraction (SPME) in the headspace (HS) (Warren, Parkinson & Pawliszyn, 2013; Kim, Park, Jang & Lee, 2011; Keles, Taskin, Baktemur, Kafkas & Büyükalaca, 2014). Clemente, Williams, Cross & Chambers (2011) identified eight sulfur volatile compounds after removing them from organic, non-organic and elephant garlic in the HS of the target vials. Despite most studies have addressed sulfur volatiles, there are many other volatile compounds in garlic that characterize its flavor and have not been studied so far. Concerning black garlic, only Kim, Park Jang & Lee (2011) have evaluated its volatiles and the influence of temperature by testing different preparation processes (autoclavage, fermentation to black garlic at a given temperature, crushing and roasting). Twenty-eight compounds were identified and relatively quantified, and only seven of them were no sulfur volatile

compounds. This study partially evaluated the behavior of volatile compounds of garlic when subjected to heating, but there are not in-depth studies on volatiles in black garlic. For this reason, a metabolomics workflow was applied to attain the following objectives: (i) to identify sulfur volatiles and flavor volatiles in black garlic; (ii) to study the evolution of the volatile profile during the fermentation process to obtain black garlic; and (iii) to monitor the changes experienced by the volatile fraction in black garlic as a function of the heating time.

2. Materials and methods

2.1. Samples and standards

Fresh garlic and black garlic from the purple garlic variety (Rocambole variety) were provided by La Abuela Carmen S.L. (Montalbán, Spain) in 2015. Obtainment of black garlic for our study involved division of fresh bulbs into cloves, peeling of the cloves and fermentation under high humidity and temperatures within 60–80 ºC in a closed chamber for 5 wk. For the study of the evolution along the fermentation process, a sample was taken off from the chamber every 6 d until day 36. The cloves were chopped and mixed, then stored at –20 ºC until analysis.

Diallyl sulfide, dimethyl trisulfide, diallyl disulfide, diallyl trisulfide, 2-butenal, 2-hexanol, benzaldehyde, 2-methylbutanal, 2-pentenal, 2,3-butanedione, 2-acetylfuran, 5-methylfurfural, 2-methylpropanal, 5-ethyl-2-methylpyridine and 2-acetylpryrrole were acquired from Sigma–Aldrich (St. Louis, USA). A multistandard solution with a concentration of 10 μg/mL of each standard in dichloromethane was prepared and analyzed with direct injection in the GC−MS.

2.2. Apparatus

Ten-mL HS vials sealed by 20 mm aluminum vial caps (Análisis Vínicos,

Tomelloso, Spain) with 20 mm silicone/PTFE septa (Análisis Vínicos) and placed in the rack of a 7694E headspace autosampler from Agilent (Palo Alto, CA, USA) were used. An Agilent 7890B gas chromatograph coupled to an Agilent 5977A mass spectrometer was used for individual separation and detection of volatile compounds. A Factor VF-5ms fused silica capillary column (30 m × 0.25 mm I.D., 0.25 μm film thickness, Varian) completed the experimental set-up.

2.3. HS–GC–MS procedure

Vial, loop, and transfer line temperatures were set at 103, 113, and 123 ºC, respectively. The vial was heated at 103 ºC for a given time without shaking, and pressurized for 0.2 min at 20.7 kPa; the loop filling and equilibration times were set at 0.2 min and 0.05 min, respectively, and the injection time was set at 0.8 min. Two g of each pool was placed in the HS vial and, after partitioning of volatiles between the solid and the headspace, 3 mL of the latter content was injected into the chromatograph. Selection of values of the HS parameters was based on the protocol optimized in our previous work (Molina-Calle, Priego-Capote & Luque de Castro, 2016). The injector temperature was fixed at 180 ºC and injection was in 1/5 split mode. The gas flow was set at 1 mL/min. The oven temperature was programmed as follows: initial temperature 40 ºC (held for 5 min), increased at 10 ºC/min to 250 ºC (held for 5 min). The total analysis time took 31 min, and 3 min extra time was necessary for re-establishing and equilibrating the initial conditions.

The single quadrupole mass spectrometer was operated in the scan mode, for which the instrumental parameters were set as follows: transfer line, source, and quad temperatures were kept at 250, 230, and 180 ºC, respectively; electron energy was set at 70 eV, data acquisition was set between *m/z* 30 and 500 and the solvent delay was set at 2 min.

2.4. Data processing and statistical analysis

Qualitative Analysis software (version 7.0, Agilent Technologies, Santa Clara, CA, USA) was used to process all data obtained by GC–MS. Treatment of raw data file started by deconvolution of potential molecular features (MFs) by the algorithm included in the software, which considered all ions exceeding 2000 counts for the absolute area parameter. The NIST Mass Spectral Search Program v. 11.0 (NIST, Washington, DC, USA) was used for spectral search (Mainlib and Replib libraries). Tentative identification was supported on correlation between experimental and database spectra above 650 in normal search mode. The retention index for each compound was calculated using a reference of linear hydrocarbons from C10 to C40, which were analyzed by the same chromatographic method. Owing to matrix effects a mismatch between the experimental and the theoretical retention time index (provided by the NIST) was detected. A correlation plot was generated to obtain a corrected RT index by extrapolation of the experimental value in the plot (see Fig. S1). Finally, the standards above indicated were used to confirm their identification by MS analysis.

Peaks corresponding to tentatively identified compounds were integrated in all samples and the resulting areas were compiled in a matrix in a .csv file. The data set was exported to Mass Profiler Professional (MPP) software package (Version 12.0, Agilent Technologies, Santa Clara, CA, USA), where the data set was treated by Principal Component Analysis (PCA) to evaluate the evolution of black garlic volatiles during fermentation and the changes in fresh and black garlic volatiles after the heating period.

3. Results and discussion

3.1. Tentative identification of volatile compounds in black garlic

The headspace of the vial containing the chopped black garlic was generated under the optimum conditions —at 103 ºC as the best temperature— and analyzed by GC–MS as described in Section 2.3. Table 1 lists the tentatively identified compounds and the main parameters that support their identification. The listed compounds are classified into three groups: derivatives from S-alk(en)yl-L-cysteine, flavor compounds, and others. The first group was composed by thirteen volatile organosulfur compounds generated through a series of chain reactions that transform S-alk(en)yl-L-cysteine (see Fig. S2). This derivative of L-cysteine is oxidized in fresh garlic, generating S-alk(en)yl-L-cysteine-S-oxides, among which the most abundant is alliin. When fresh garlic is chopped, the enzyme alliinase is activated and transforms alliin and the rest of S-alk(en)yl-L-cysteine-S-oxides into sulfenic acid intermediates. In the aqueous cell medium, the intermediates are rapidly condensed into thiosulfinates, among which the derivative from alliin is allicin, the major thiosulfinate in fresh garlic. These compounds are very unstable and give rise to further reactions leading to a wide variety of sulfur derivatives, most of them volatile (Lanzotti, 2006).

The sulfur volatiles in black garlic presented a similar fragmentation pattern, as can be seen in Table 1, where the most intense fragments of each compound are listed. Allyl methyl sulfide was the most intense compound in black garlic within this family, reaching a relative concentration above 18%. The remaining sulfur compounds identified in the sample provided a relative concentration below 2%, being allyl methyl trisulfide the second most concentrated (1.5%). No hydrogen sulfide was detected, possibly because the small difference in temperature between the fermentation and sampling processes as compared with the time garlic is subjected to each process.

Table 1 Volatile compounds tentatively identified from the headspace generated from fresh and black garlic and their concentration in the samples.

Compound	RT (min)	RT Index	Theoretical RT Index	Match	Mass (Da)	Formula	Principal fragments	Fresh garlic (%)	Black garlic (%)
Derivatives from S-alk(en)yl-L-cysteine									
Dimethyl sulfide	1.67	553	455	813	58.0	C_4H_6O	32.0, 41.0, 43.0, 56.0	0.06	–
Allyl mercaptan	2.07	574	562	817	74.0	C_3H_6S	39.1, 41.1, 72.1, 74.0	1.4	1.1
Allyl methyl sulfide	3.12	629	660	813	88.0	C_4H_8S	39.0, 45.0, 73.0, 88.0	3.6	18.2
Dimethyl disulfide	4.07	679	722	892	94.0	$C_2H_6S_2$	45.0, 55.0, 84.0, 93.9	1.2	0.65
Diallyl sulfide	7.42	854	849	820	114.1	$C_6H_{10}S$	39.0, 45.0, 73.0, 114.0	4.8	1.4
Allyl n-propyl sulfide	7.81	875	859	828	116.1	$C_6H_{12}S$	32.0, 43.0, 44.0, 55.0	–	0.15
Allyl 1-propenyl sulfide	8.18	894			114.1	$C_6H_{10}S$	32.0, 45.0, 99.0, 114.0	0.06	–
Di(1-propenyl) sulfide	8.27	899	884	750	114.1	$C_6H_{10}S$	32.0, 41.0, 45.0, 99.0	0.16	–
1,3-Dithiane	8.85	912	1002	768	120.2	$C_4H_8S_2$	39.0, 41.0, 45.0, 119.9	5.5	0.92
Allyl methyl disulfide	9.08	941	911	654	120.0	$C_4H_8S_2$	32.0, 41.0, 72.0, 115.9	–	–
Methyl 1-propenyl disulfide	9.43	960	928	750	120.0	$C_4H_8S_2$	45.0, 72.0, 75.0, 119.9	0.33	–
Dimethyl trisulfide	10.09	995	972	902	126.0	$C_2H_6S_3$	45.0, 63.9, 78.9, 125.9	0.73	1.0
Diallyl disulfide	12.25	1108	1099	865	146.0	$C_6H_{10}S_2$	39.0, 41.1, 81.0, 146.0	21.0	0.84
Allyl 1-propenyl disulfide	12.52	1122			146.0	$C_6H_{10}S_2$	41.1, 45.0, 73.0, 146.0	0.90	–
Di(1-propenyl) disulfide	12.63	1127	1103	816	146.0	$C_6H_{10}S_2$	41.1, 45.0, 73.0, 146.0	2.6	–
Allyl methyl trisulfide	13.28	1162	1161	656	152.0	$C_4H_8S_3$	41.0, 45.0, 73.0, 87.0	8.8	1.5
3-Vinyl-1,2-dithiacyclohex-4-ene	14.10	1205	1134	778	144.0	$C_6H_8O_2$	79.0, 97.0, 111.0, 144.0	0.26	–
3-Vinyl-1,2-dithiacyclohex-5-ene	14.48	1224	1155	900	144.0	$C_6H_8O_2$	71.0, 72.0, 111.0, 144.0	0.92	0.13
Benzothiazole*	14.66	1234	1208	863	135.0	C_7H_5NS	32.0, 44.0, 108.0, 134.9	–	0.33
Diallyl trisulfide	15.72	1292	1350	854	178.0	$C_6H_{10}S_3$	39.0, 41.1, 73.0, 113.0	21.3	0.86
Di(1-propenyl) trisulfide	16.15	1312			178.0	$C_6H_{10}S_3$	41.0, 45.0, 73.0, 114.0	0.21	0.11
Diallyl tetrasulfide	18.95	1459	1510	812	210.0	$C_6H_{10}S_4$	39.0, 41.0, 73.0, 146.0	0.06	–

Cont. Table 1

Flavor compounds									
Green/Floral flavor									
2-Butenal	2.53	598	615	943	70.0	C_4H_6O	39.0, 41.0, 69.0, 70.0	12	–
3-Penten-2-one*	3.98	674	714	799	84.1	C_5H_9O	41.0, 43.0, 69.0, 84.0	0.04	0.42
2-Methyl-4-pentenal	4.19	685	732	796	98.1	$C_6H_{10}O$	41.0, 43.0, 45.0, 55.0	–	–
2-Hexanol*	4.38	695	780	720	102.1	$C_6H_{14}O$	39.0, 55.0, 83.0, 84.0	0.25	–
5-Hexen-2-one*	5.01	728	744	908	98.1	$C_6H_{10}O$	39.0, 43.0, 55.0, 83.0	0.18	–
5-Hexenal*	5.35	746	796	875	98.1	$C_6H_{10}O$	39.0, 41.0, 54.0, 80.0	0.29	0.29
Hexanal*	5.74	767	803	890	100.1	$C_6H_{12}O$	41.0, 44.0, 56.1, 72.0	0.28	0.16
4-Heptenal	7.09	837	870	840	112.1	$C_7H_{12}O$	41.0, 53.0, 67.0, 68.0	1.0	–
2,4-Hexadienal	8.77	925	877	833	96.1	C_6H_8O	53.0, 67.0, 96.0, 81.0	0.74	–
Benzaldehyde*	9.91	985	920	909	106.0	C_7H_6O	51.0, 77.0, 92.0, 106.0	–	0.62
Benzeneacetaldehyde	11.60	932.1	1081	953	120.1	C_8H_8O	65.0, 91.0, 92.0, 120.0	–	12
3-Methyl-benzaldehyde*	12.05	1097	1060	884	120.1	C_8H_8O	65.0, 91.0, 119.0, 120.0	–	–
Roasted flavor									
3-Methyl butanal*	2.59	601	643	930	86.0	$C_5H_{10}O$	39.0, 41.0, 69.0, 70.0	0.48	8.8
2-Methyl butanal*	2.70	607	643	912	86.0	$C_5H_{10}O$	41.0, 43.0, 57.1, 58.0	–	3.0
2-Pentenal	2.96	621	697	885	84.1	C_5H_8O	39.0, 41.0, 55.0, 83.0	–	–
Dihydro-2-methyl-3-furanone	5.99	468.1	821	850	100.1	$C_5H_8O_2$	32.0, 43.0, 72.0, 100.0	–	–
Furfural*	6.72	818	831	960	96.0	$C_5H_4O_2$	39.0, 67.0, 95.0, 96.0	–	17.3
3-Ethyl pyridine*	9.86	982	937	879	107.1	C_7H_9N	51.0, 77.0, 105.0, 106.0	–	–
Sweet flavor									
2,3-Butanedione*	2.06	143.4	558	805	86.0	$C4H_6O_2$	39.0, 41.1, 45.0, 74.0	–	0.91
1-Hydroxy-2-propanone	3.72	280.7	698	832	74.0	$C_3H_6O_2$	31.0, 43.0, 45.0, 74.0	–	0.74
2-Acetylfuran*	8.80	700.6	878	943	110.0	$C_6H_6O_2$	39.0, 42.0, 81.0, 108.0	–	0.15
5-Methylfurfural*	10.00	799.7	920	909	110.0	$C_6H_6O_2$	53.0, 81.0, 109.0, 110.0	–	0.53
Various flavor									
2-Methylpropanal* (spicy)	1.91	131.1	543	829	72.1	C_4H8O	39.1, 41.1, 43.1, 72.1	–	2.8
2-Ethyl-2-butenal (pungent)	6.30	494.1	791	935	98.1	$C_6H_{10}O$	41.0, 55.0, 69.0, 98.0	1.9	–
5-Ethyl-2-methyl-pyridine* (nutty)	11.21	900.2	1000	845	121.1	$C_8H_{11}N$	45.0, 77.0, 106.0, 121.0	–	–
2-Acetylpirrole* (musty)	11.94	960.6	1035	821	109.1	C_6H_7NO	39.0, 66.0, 94.0, 109.0	–	–
Other compounds									
Acetone*	1.67	569	455	813	58.0	C_3H_6O	40.0, 41.0, 43.0, 56.0	1.8	6.4
2-Propen-1-ol	1.86	579	552	945	58.0	C_3H_6O	31.1, 39.1, 57.0, 58.0	5.3	0.82
2-Methylene-4-pentenal	4.26	689	763	829	96.1	C_6H_8O	39.0, 41.0, 67.0, 95.0	0.29	14.9

The asterisk indicates compounds tentatively identified for the first time.

Tentatively identified compounds that provide flavor to black garlic constituted the second group and they were classified as a function of their flavor. Five compounds with green or floral flavors, and three compounds with roasted flavor were identified in the headspace from black garlic samples; among the latter, furfural (which presents a roasted almond flavor) was the most remarkable, reaching a relative concentration above 17%. This compound, characteristic of the Maillard reaction, results from degradation of a pentose sugar. Two derivatives from furfural were also identified in the analytical sample generated in the headspace: 2-acetylfuran and 5-methylfurfural; all of them are also generated in the Maillard reaction and associated to a characteristic sweet flavor, as do 2,3-butanedione and 1-hydroxy-2-propanone. Finally, 2-methylpropanal, with a spicy flavor, was also tentatively identified in the sampled headspace.

The last group encompasses three compounds without flavor characteristics (*viz.,* acetone, 2-propen-1-ol, and 2-methylene-4-pentenal), which were also tentatively identified in the content of the headspace from black garlic; all them with a high volatility as corresponds to their appearance within the first 4 min of the chromatogram.

The identity of the compounds marked in Table 1 with an asterisk was assessed by analysis of the corresponding standards. The spectra of diallyl sulfide, dimethyl trisulfide, diallyl disulfide and diallyl trisulfide detected in the samples can be compared to those provided by the standards in Fig. S3 to corroborate the tentative identification.

3.2. Compositional differences between black garlic and fresh garlic

Black garlic presented significant differences in the volatile profile regarding to fresh garlic, as can be seen in Table 1. First, the relative concentration of derivatives of S-alk(en)yl-L-cysteine in black garlic constituted less than 28% of the total relative concentration estimated as the sum of peaks area from all the

identified compounds. This value represented a reduction of 42% with respect to its relative concentration in fresh garlic, which reached up to 70%. Furthermore, some of the sulfur volatiles quantified in fresh garlic (*viz.*, dimethyl sulfide, allyl 1-propenyl sulfide, di(1-propenyl) sulfide and 3-vinyl-1,2-dithiacyclohex-4-ene) were not detected in black garlic. Diallyl trisulfide was the major sulfur volatile in fresh garlic, but its percentage in black garlic was around 1%. On the contrary, allyl methyl sulfide was very concentrated in black garlic (18%), and below 4% in fresh garlic.

The differences between fresh and black garlic were especially remarkable in the flavor volatile profile. Fresh garlic was richer in green and floral flavor compounds, with special emphasis on 2-butenal that presented the highest relative concentration within the group of flavor compounds in this sample (above 12%). On the other hand, benzeneacetaldehyde, the second most concentrated volatile in black garlic, was not detected in fresh garlic. Roasted and sweet flavor compounds were clearly characteristic of black garlic since only 3-methylbutanal was detected in fresh garlic. In black garlic, furfural was the most concentrated flavor compound, reaching a concentration above 27% as a consequence of development of the Maillard reaction during fermentation. Concerning compounds with other flavors, only 2-ethyl-2-butenal, with a pungent flavor, was detected in fresh garlic, while 2-methylpropanal, with a spicy flavor, was the only one detected in black garlic. Finally, within the group of other compounds, black garlic showed to be richer in acetone and 2-methylene-4-pentenal than fresh garlic, while the latter provided a higher concentration in 2-propen-1-ol.

3.3. HS–GC–MS monitoring of black garlic production

Purple garlic was fermented and the fermenter content was sampled every 6 d during the process for subsequent analysis by the proposed HS–GC–MS method. In overall terms, the analysis of the different fractions revealed that during fermentation the derivatives from S-alk(en)yl-L-cysteine gradually decreased their

relative concentration until day 36. The concentration profile of the two major organosulfur volatiles at day 0, diallyl disulfide and diallyl trisulfide, was characterized by an intense decrease during the first 6 d, as can be seen in Fig. 1. A similar behavior was that of compounds with representative green or floral flavor, which were only detected in the sample collected prior to starting fermentation. A particular example is illustrated in Fig. 1 for 3-methyl-benzaldehyde that was not detected in the samples collected after 6 fermentation days, probably owing to the combined effect of temperature and humidity. 2-Ethyl-2-butenal followed a behavior similar to 3-methyl-benzaldehyde, since it was not detected after 6 fermentation days; while sweet and roasted flavor compounds exhibited an opposite behavior: their relative concentration increased along the fermentation process. This is the case for furfural, which was not detected at the beginning of the process, but during fermentation experienced an exponential growth up reaching a relative concentration of 24.7% at day 36 (see Fig. 1). Similarly, 2-acetylfuran and benzaldehyde were not detected until day 30. As mentioned before, furfural and 2-acetylfuran are generated in the Maillard reaction and their appearance or increased concentration during the last days of fermentation suggests degradation of sugars present in garlic, favored by the fermentation conditions.

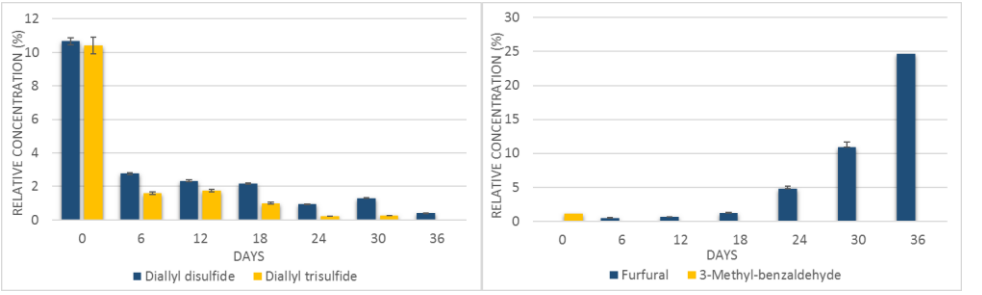

Figure 1. Evolution of the relative concentration (expressed as % of the total peak area) of diallyl disulfide, diallyl trisulfide, furfural and 3-methyl-benzaldehyde during black garlic fermentation.

Finally, the compounds with low molecular mass and high volatility identified in fresh garlic (acetone, 2-propen-1-ol and 2-methylene-4-pentenal) were relatively quantified in all the samples collected during monitoring of the fermentation process. They experienced a particular behavior, with maximum concentration at intermediate fermentation times and decreasing during the last days of the process: a behavior that was more significant for 2-propen-1-ol.

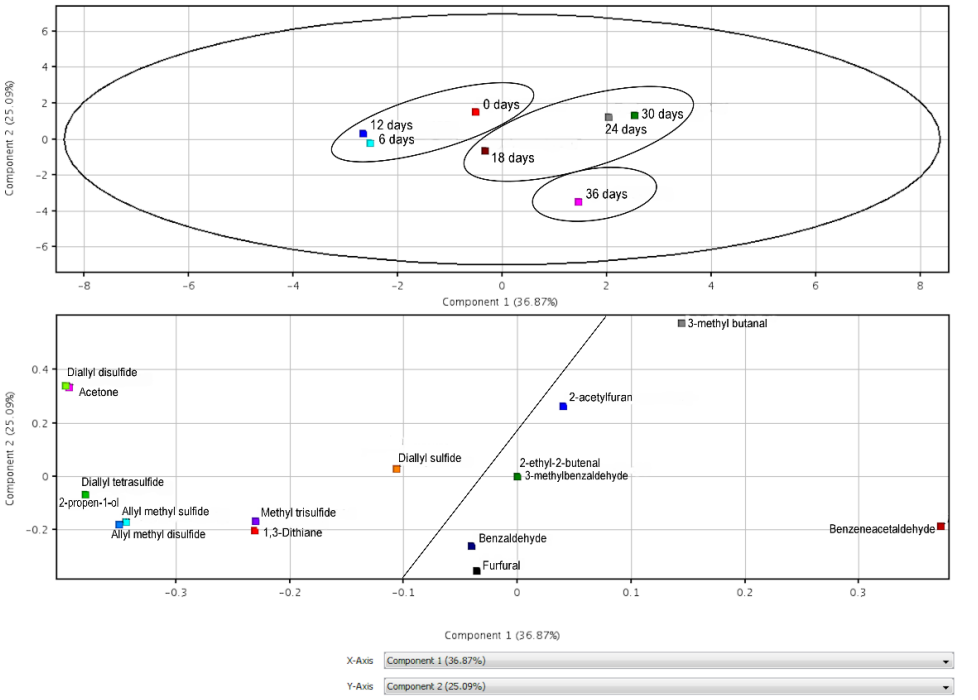

Figure 2. PCA scores and loadings plots describing the evolution of garlic during fermentation and the contribution of tentatively identified compounds to the variability among samples.

The data obtained from the fermentation study were firstly screened by selecting those compounds that experienced the most significant alterations during fermentation for statistical analysis by PCA as described in the experimental section. The resulting PCA scores and loadings graphs are shown in

Fig. 2. The former PCA allows distinguishing two groups of samples as a function of Principal Component 1 (PC1) and 2 (PC2), which explain more than 62% of the variance among samples. A clear separation according to the fermentation time was observed along PC1 with contribution of PC2. The loadings plot shows a clear division of the compounds into two groups according to the discrimination trend observed in the scores plot. Compounds on the left side included derivatives from S-alk(en)yl-L-cysteine, acetone and 2-propen-1-ol, responsible for differentiation of the samples until day 18. The group of compounds on the right side was exclusively formed by flavor components, benzeneacetaldehyde being the compound present that explained the highest variability along PC1. Thus, the main changes occurring in the first half interval of the fermentation process were associated to the organosulfur fraction, while alterations in the second half affected to flavor components.

3.4. Heating kinetics of fresh and black garlic

Black and fresh garlic were subjected to heating in the autosampler at 103 ºC during different times (10, 20, 30, 40, 50, 60, 90 and 120 min) to evaluate compositional changes in the volatile profile. Concerning the S-alk(en)yl-L-cysteine derivatives, a significant decrease of their concentration (10% of the total area) was detected in fresh garlic when subjected to 120 min heating, while a slight increase of 0.4% was produced in the case of black garlic when heated during the same interval. The behavior of diallyl mono-, di, tri- and tetrasulfide can be taken as a model of that of sulfur volatiles in fresh and black garlic (see Fig. 3). The relative concentration of diallyl di- and trisulfide in fresh garlic slighly decreased after 120 min heating, losing in the process 11 and 5% of their relative concentration, respectively. On the contrary, the relative concentration of diallyl mono- and tetrasulfide experienced a significant increase during heating: the former triplicated its concentration after 120 min heating, while the latter achieved a concentration five times higher at min 10. The relative concentration of diallyl

sulfide in black garlic decreased during the first 20 min of heating, then increased to reach the initial concentration; diallyl tetrasulfide decreased its relative concentration that became undetected after 120 min heating (see Table S1). On the contrary, diallyl disulfide and diallyl trisulfide increased about twice their relative percentage in black garlic at the end of the heating time.

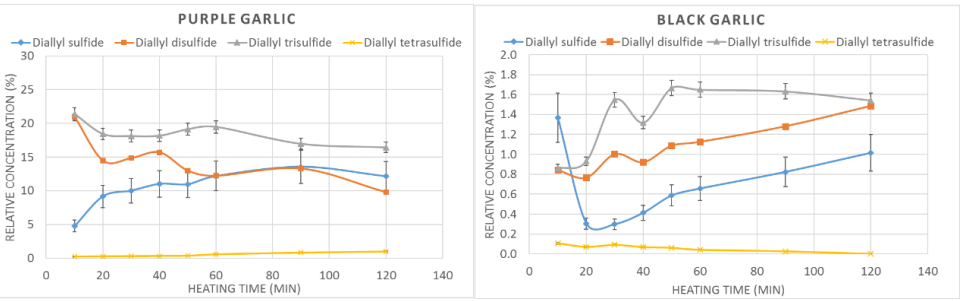

Figure 3. Evolution of the relative concentration (percentage of the total peak area) of diallyl sulfide, diallyl disulfide, diallyl trisulfide and diallyl tetrasulfide in black garlic and fresh garlic during the heating time.

Clear differences in the behavior of flavor compounds were observed between the two types of garlic. Concerning the green or floral flavor group, all compounds tentatively identified were quantified in fresh garlic, excluding benzeneacetaldehyde, which was the most concentrated compound in black garlic within this group at any heating time. This compound constituted 11% of the total peak area after 10 min heating; then, the percentage was gradually reduced for longer heating times. Black garlic, however, was much poorer in this variety of compounds than fresh garlic as only 5 compounds were quantified with this flavor and all of them decreased their concentration along the heating time (see Fig. S4). 2-Butenal was the major flavor volatile in fresh garlic after the first 10 min heating, but its concentration drastically decreased along the heating kinetics, probably due to its high volatility. Complete volatilization can be the reason why this compound was not detected in black garlic. On the other hand, benzaldehyde and 3-methylbenzaldehyde appeared in fresh garlic after 20 min heating. The flavor of

benzeneacetaldehyde, benzaldeyde and 3-methylbenzaldehyde is listed as green or floral, but characterized by honey and sweet notes, which could justify the behavior of these aldehydes as more similar to sweet flavor compounds than to green or floral compounds. Nevertheless, benzaldehyde and benzeneacetaldehyde decreased their relative concentration in black garlic along the heating time, especially benzeceacetaldehyde that loss about 6% of its relative concentration after 20 min heating (see Fig. S4).

Concerning roasted and sweet flavor compounds, black garlic was clearly richer in them than fresh garlic: 3-methyl butanal was the only of these compounds detected in fresh garlic after 10 min heating, while among sweet flavor compounds only 1-hydroxy-2-propanone was detected in this sample at min 60, increasing its concentration up to 0.23% at the end of the 120 min heating period. The most concentrated flavor compound in black garlic was furfural, with clear predominance in the volatile profile. Together with furfural, the behavior of dihydro-2-methyl-3-furanone, 2-acetylfuran and 5-methyl furfural, products of the Maillard reaction, can be considered as a model of the evolution for this reaction along the heating kinetics. Fig. S3 shows the evolution of the relative concentration of furfural, 2-acetylfuran and 5-methyl furfural in black garlic along the heating time. The relative concentration of furfural increased almost 50% until min 50, decreasing after this time, with a final balance of an increase slightly above 10%. The increase of 2-acetylfuran and 5-methylfuranone was progressive, reaching a relative concentration, as compared with the initial, ten and two times higher, respectively, at the final of the heating kinetics. Moreover, dihydro-2-methyl-3-furanone was only detected in black garlic after 90 min heating. Regarding compounds with other flavor, 2-ethyl-2-butenal and 5-ethyl-2-methylpyridine were relatively quantified in fresh garlic but not detected in black garlic. As previously assumed, these compounds are indicative of garlic freshness; therefore, their disappearance during fermentation was foreseeable. On the other

hand, 2-methyl propanal and 2-acetylpirrole (with spicy and musty flavor) were only detected in black garlic. The former compound increased its relative concentration at longer heating times, meanwhile the latter appeared in the headspace at min 40.

Finally, among the rest of compounds, 2-propen-1-ol was the most concentrated in fresh garlic and its concentration drastically increased after 20 min heating and the increase continued until reaching more than 24% of the total relative concentration at min 120. Among this group, 2-methylene-4-pentenal provided the highest concentration in black garlic (more than 14% at min 10), although it decreased to 4% after 120 min heating.

3.5. Principal Component Analysis of black garlic and fresh garlic under heating

Two data sets, one for each type of garlic, were created from the data obtained as described under Experimental and treated by PCA to discriminate among heating times and establish the compounds with the largest contribution to separation. The obtained PCAs are shown in Fig. 4. In the PCA from black garlic, the resulting PC1 is the most significant since it explained 61.6% of the total variability and is responsible for the separation of the samples in the plot (particularly noticeable is the separation of the sample heated for 10 min). 3-Vinyl-1,2-dithiacyclohex-5-ene and benzothiazole are the only compounds with a positive value in this Component; therefore, their concentration differentiates black garlic heated during the first 10 min from the rest of heating times. In contrast, methyl allyl disulfide, dimethyl disulfide and furfural provided the lowest values in PC1; thus, these compounds clearly influence the separation of the samples with the highest heating time in the left side of the plot. Also PC2 influenced separation between samples subjected to heating times longer than 10 min. In this case, diallyl tetrasulfide and 2,3-butanedione presented the highest value in this component, while allyl methyl sulfide, diallyl sulfide and methyl 1-propenyl disulfide provided the lowest, being responsible for the separation of the

samples heated for 90 and 120 min. Despite flavor compounds are of importance to characterize the aroma of black garlic and heated black garlic, they lose significance, regarding sulfur compounds, for sample discrimination when they are treated statistically by PCA.

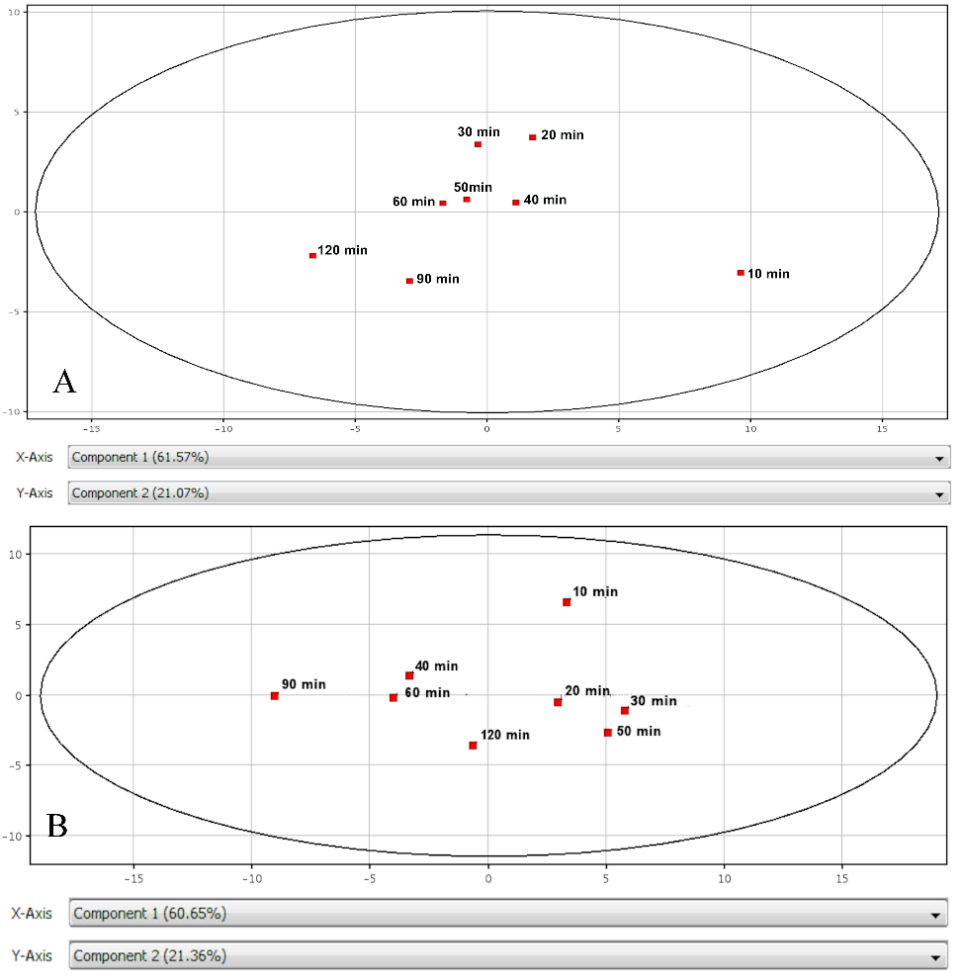

Figure 4. PCA of black (A) and fresh (B) garlic samples subjected to different heating times.

Regarding fresh garlic, PC1 also explained the highest percentage of variability (more than 60%), although only separation of the samples heated

between 20 and 90 min is explained. PC2 determined the separation between the first point (10 min heating), the intermediate points (20−90 min) and the final point (120 min) of the heating kinetics. In this component, 2-butenal and 3-methylbutanal presented the highest values; thus, they were responsible for the separation of garlic heated for 10 min. On the other hand, 2-propen-1-ol, 4-heptenal and 2-methyl-4-pentenal provided the lowest values, leading to the separation of garlic heated for 120 min. The separation between the intermediate heating times was clearly produced along PC1, where allyl mercaptane and 1-hydroxy-2-propanone provided the highest values and influenced the separation of the garlic heated for the shortest times. Dimethyl disulfide, allyl trisulfide and 1-propenyl allyl disulfide presented the lowest values in PC1, being responsible for differentiation between short (up to 30 min) and long heating times (up to 120 min). Thus, flavor compounds, together with those that did not provide flavor, allow discrimination between the first and final point of the fresh garlic heating kinetics; while sulfur volatiles induced a trend in the heating kinetics: the longer the heating time, the more shifted to the right will be the sample in the PCA.

4. Conclusions

An optimized method based on HS−GC–MS was used to study the volatile profiles along fermentation of black garlic in comparison with fresh garlic (both of the purple variety), and their behavior under heating. A total of 51 volatiles were identified in the HS generated from all the samples under study. First, the comparison of fresh and black garlic volatile profiles showed that the former provided a higher concentration in sulfur volatiles; however, the main differences were found in the flavor volatiles. The study of the volatile profile of black garlic along fermentation revealed that the process decreased the concentration of the sulfur volatiles, while highly increased those of sweet and roasted volatiles, especially furfural and its derivatives, due to the promotion of the Maillard

reaction. These results provide new information about the aroma constituents of black garlic, whose volatile profile had not been characterized so far, and how this volatile profile is transformed due to fermentation from fresh to black garlic, suggesting some of the reactions that take place during the process. Finally, when fresh and black garlic were subjected to different heating times, the main sulfur volatiles presented an opposite behavior along heating: in both types of garlic the concentration on green and floral volatiles decreased along heating, while sweet and roasted flavor volatiles increased their concentration, especially in black garlic, which achieved more than 50% of the total area of these compounds. Black garlic is gaining popularity in cuisine but no studies have been addressed to evaluate the effect of cooking on its composition; thus, this last study allows modelling the behavior of fresh and black garlic when they are cooked, revealing the different aroma they can provide to meals.

Acknowledgements

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Supplementary information

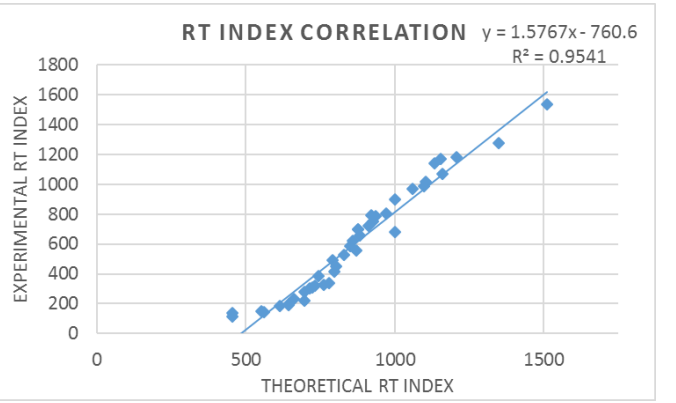
Supplementary Table 1 Relative concentration (as percentage of the sum of the peak areas) of compounds tentatively identified in the samples of fresh and black garlic heated at 103 ºC in HS vials for different times.

Compound	Fresh garlic							
	10 min	20 min	30 min	40 min	50 min	60 min	90 min	120 min
Derivatives from S-alk(en)yl-L-cysteine								
Dimethyl sulfide	0.06	0.09	0.13	0.18	0.09	0.08	0.14	0.08
Allyl mercaptane	1.4	0.98	0.56	0.18	0.22	0.13	0.11	0.17
Allyl methyl sulfide	3.6	9.1	6.4	5.8	8.2	6.3	6.2	5.4
Dimethyl disulfide	1.2	1.2	1.2	1.5	1.1	1.3	1.5	1.4
Diallyl sulfide	4.8	9.2	10.0	11.0	10.9	12.0	13.6	12.1
Allyl n-propyl sulfide	–	–	–	–	–	–	–	–
Allyl 1-propenyl sulfide	0.06	0.13	0.11	0.14	0.12	0.15	0.13	0.11
Di(1-propenyl) sulfide	0.16	0.16	0.12	0.15	0.11	0.13	0.08	0.05
1,3-Dithiane	5.4	2.2	2.1	2.7	1.7	0.46	2.6	2.2
Allyl methyl disulfide	–	0.75	0.07	0.08	0.06	0.01	0.13	0.20
Methyl 1-propenyl disulfide	0.33	0.37	0.38	0.51	0.40	0.46	0.51	0.49
Dimethyl trisulfide	0.73	0.65	0.63	0.87	0.70	0.89	1.2	1.4
Diallyl disulfide	21.0	14.6	14.8	15.7	13.0	12.1	13.3	9.8
Allyl 1-propenyl disulfide	0.90	0.79	0.69	0.94	0.66	0.75	0.82	0.61
Di(1-porpenyl) disulfide	2.6	2.5	2.2	2.9	2.2	2.3	2.3	1.7
Methyl 2-propenyl trisulfide	8.8	7.7	7.7	8.7	8.4	9.2	8.9	10.1
3-Vinyl-1,2-dithiacyclohex-4-ene	0.26	0.15	0.20	0.16	0.15	0.16	0.21	0.13
3-Vinyl-1,2-dithiacyclohex-5-ene	0.92	0.54	0.74	0.49	0.56	0.51	0.61	0.40
Benzothiazole	–	–	–	–	–	0.07	–	–
Diallyl trisulfide	21.3	18.6	18.1	18.2	19.1	19.2	17.0	16.5
Diallyl tetrasulfide	0.21	0.26	0.28	0.33	0.35	0.57	0.84	1.01
Flavor compounds								
Green/Floral flavor								
2-Butenal	12.2	1.4	0.84	1.9	1.0	1.2	0.67	0.19
3-Penten-2-one	0.04	0.07	0.06	0.12	0.04	0.10	0.13	0.05
2-Methyl-4-pentenal	0.25	0.54	0.59	0.70	0.69	0.82	1.0	0.94
2-Hexanol	–	–	–	–	0.08	0.04	0.04	0.10
5-Hexen-2-one	0.18	0.06	0.06	0.09	0.05	0.12	0.08	0.08
5-Hexenal	0.29	1.3	1.1	1.5	1.0	1.4	1.3	0.89
Hexanal	0.28	0.09	0.06	0.19	0.05	0.09	0.10	0.04
4-Heptenal	1.0	4.1	3.8	5.4	3.8	6.2	6.5	5.4
2,4-Hexadienal	0.74	0.09	0.12	0.11	0.05	0.05	–	–
Benzaldehyde	–	0.11	0.25	0.14	0.19	0.19	0.19	0.18
Benzeneacetaldehyde	–	–	–	–	–	–	–	–
3-Methyl-benzaldehyde	–	0.12	0.15	0.10	0.15	0.18	0.15	0.19
Roasted flavor								
3-Methylbutanal	0.48	0.06	0.07	0.09	0.05	0.06	0.06	0.04
2-Methylbutanal	–	–	–	0.03	0.03	0.03	0.04	–
2-Pentenal	–	0.22	0.20	0.20	0.25	0.26	0.19	0.18
Dihydro-2-methyl-3-furanone	–	–	–	–	–	–	–	–

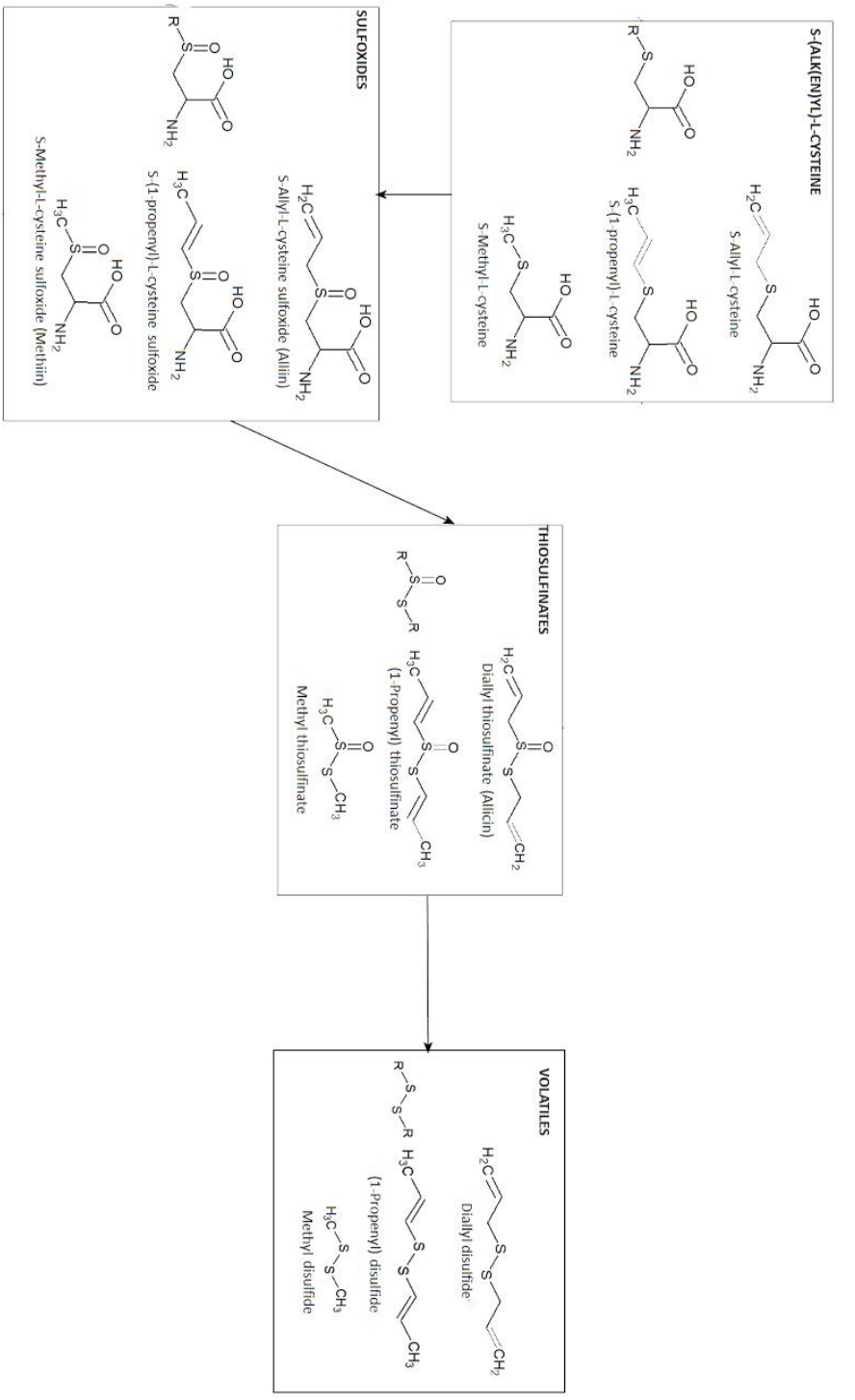
Furfural	–	0.08	0.08	0.05	0.11	0.08	0.08	0.12
3-Ethylpyridine	–	–	–	–	–	0.07	0.14	0.13
Sweet flavor								
2,3-Butanedione	–	–	–	–	–	–	–	–
1-Hydroxy-2-propanone	–	–	–	–	–	0.06	0.05	0.23
2-Acetylfuran	–	–	–	–	–	–	–	–
5-Methylfurfural	–	–	–	–	–	–	–	–
Other flavors								
2-Methyl propanal (spicy)	–	–	–	–	–	–	–	–
2-Ethyl-2-butenal (pungent)	1.9	0.59	0.76	1.0	0.50	0.87	0.57	0.26
5-Ethyl-2-methyl-pyridine (nutty)	–	0.05	0.14	0.09	0.07	0.12	0.20	0.23
2-Acetylpirrole (musty)	–	–	–	–	–	–	–	–
Other compounds								
Acetone	1.9	2.7	2.8	3.0	2.0	2.3	2.3	2.0
Methylene chloride	1.2	0.19	0.27	0.71	0.08	0.37	0.20	0.07
2-Propen-1-ol	5.3	18.6	21.6	13.5	21.2	16.7	15.8	24.8
2-Methylene-4-pentenal	0.29	0.49	0.51	0.42	0.45	0.47	0.21	0.08

Compound	**Black garlic**							
	10 min	**20 min**	**30 min**	**40 min**	**50 min**	**60 min**	**90 min**	**120 min**
Compounds derived from S-alk(en)yl-L-cysteine								
Dimethyl sulfide	–	–	–	–	–	–	–	–
Allyl mercaptane	1.1	1.9	1.6	1.8	1.9	1.6	2.3	1.6
Allyl methyl sulfide	18.2	1.10	1.2	1.2	1.8	2.4	2.8	4.0
Dimethyl disulfide	0.65	1.9	2.0	1.7	1.9	2.2	2.4	3.1
Diallyl sulfide	1.4	0.30	0.30	0.41	0.59	0.66	0.82	1.0
Allyl n-propyl sulfide	0.15	0.05	0.09	0.18	0.12	0.06	0.08	0.05
Allyl 1-propenyl sulfide	–	–	–	–	–	–	–	–
Di(1-propenyl) sulfide	–	–	–	–	–	–	–	–
1,3-Dithiane	0.92	2.2	2.7	2.3	2.6	2.8	2.9	3.2
Allyl methyl disulfide	0.01	0.03	0.10	0.12	0.11	0.09	0.09	0.10
Methyl 1-propenyl disulfide	–	0.04	0.07	0.06	0.07	0.08	0.08	0.07
Dimethyl trisulfide	1.0	3.3	5.0	3.9	4.5	4.5	4.5	4.5
Diallyl disulfide	0.84	0.77	1.0	0.92	1.1	1.1	1.3	1.5
Allyl 1-propenyl disulfide	–	–	0.02	0.03	0.03	0.03	0.03	0.03
Di(1-porpenyl) disulfide	–	–	0.03	0.03	0.04	0.04	0.04	0.04
Methyl 2-propenyl trisulfide	1.5	4.3	7.0	5.7	7.1	7.1	7.0	6.9
3-Vinyl-1,2-dithiacyclohex-4-ene	–	–	–	–	–	–	–	–
3-Vinyl-1,2-dithiacyclohex-5-ene	0.13	–	–	–	–	–	–	–
Benzothiazole	0.33	–	–	–	–	–	–	–
Diallyl trisulfide	0.86	0.93	1.6	1.3	1.7	1.7	1.6	1.5
Diallyl tetrasulfide	0.11	0.07	0.09	0.07	0.06	0.04	0.03	–
Flavor compounds								
Green/Floral flavor								
2-Butenal	–	–	–	–	–	–	–	–
3-Penten-2-one	0.42	0.17	0.23	0.23	0.21	0.15	0.12	0.09

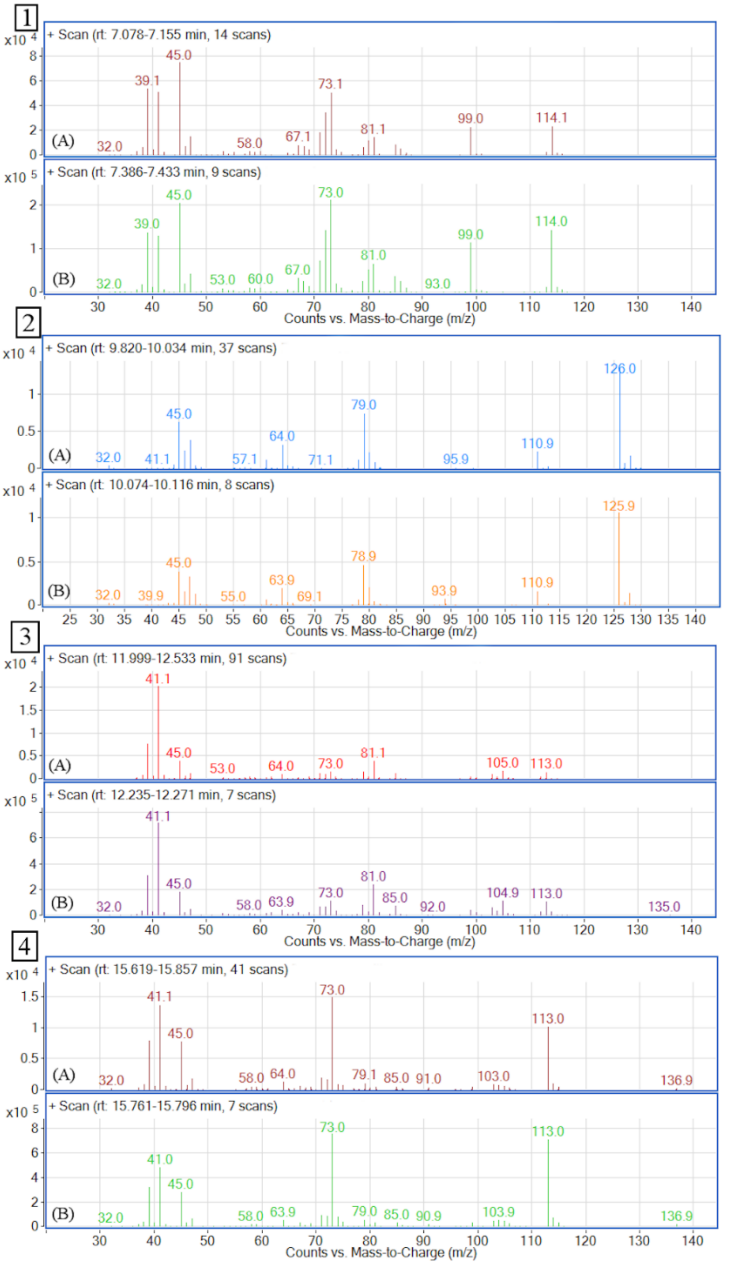
2-Methyl-4-pentenal	–	–	–	–	–	–	–	–
2-Hexanol	–	–	–	–	–	–	–	–
5-Hexen-2-one	–	–	–	–	–	–	–	–
5-Hexenal	0.29	0.18	0.22	0.18	0.18	0.14	0.10	0.08
Hexanal	0.16	0.18	0.20	0.12	0.13	0.13	0.10	0.12
4-Heptenal	–	–	–	–	–	–	–	–
2,4-Hexadienal	–	–	–	–	–	–	–	–
Benzaldehyde	0.62	0.75	0.60	0.48	0.54	0.49	0.39	0.38
Benzeneacetaldehyde	12	5.3	6.0	5.3	4.9	3.4	2.8	1.9
3-Methyl-benzaldehyde	–	–	–	–	–	–	–	–
Roasted flavor								
3-Methylbutanal	8.8	21	17	22	15	23	19	24
2-Methylbutanal	3.0	6.7	6.4	7.2	6.5	7.8	7.3	8.6
2-Pentenal	–	–	–	–	–	–	–	–
Dihydro-2-methyl-3-furanone	–	–	–	–	–	–	0.09	0.12
Furfural	17.3	19.3	22.2	21.7	25.5	20.0	24.2	19.3
3-Ethylpyridine	–	–	–	–	–	–	–	–
Sweet flavor								
2,3-Butanedione	0.91	1.3	0.66	0.53	0.55	0.45	0.34	0.27
1-Hydroxy-2-propanone	0.74	0.05	0.29	1.2	0.60	0.11	1.1	0.95
2-Acetylfuran	0.15	0.42	0.44	0.62	0.72	0.74	1.2	1.4
5-Methylfurfural	0.53	0.50	0.65	0.69	0.80	0.69	1.0	1.1
Other flavors								
2-Methyl propanal (spicy)	2.8	5.9	5.2	4.7	4.6	5.0	4.5	5.0
2-Ethyl-2-butenal (pungent)	–	–	–	–	–	–	–	–
5-Ethyl-2-methyl-pyridine (nutty)	–	–	–	–	–	–	–	–
2-Acetylpirrole (musty)	–	–	–	–	0.05	0.05	0.07	0.08
Other compounds								
Acetone	6.4	7.3	6.6	6.9	7.6	5.9	6.7	4.9
Methylene chloride	2.9	1.7	1.1	0.85	0.58	0.60	0.16	0.22
2-Propen-1-ol	0.82	1.4	0.46	0.38	0.35	0.43	0.11	0.15
2-Methylene-4-pentenal	14.9	11.0	9.3	7.2	7.4	6.3	4.8	4.0



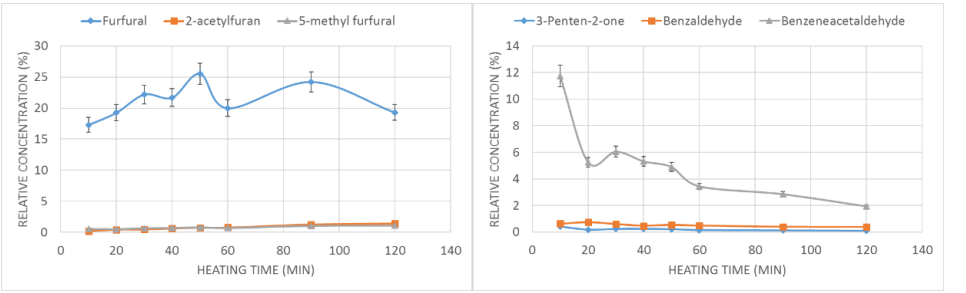
Supplementary Figure 1. Correlation plot between the theoretical RT index (provided by the NIST) and the experimental RT index (calculated by a regression model using an alkanes mixture).



Supplementary Figure 2. Metabolic pathway of the organosulfur compounds in garlic giving place to the sulfur volatiles.



Supplementary Figure 3. Spectra of diallyl sulfide (1), dimethyl trisulfide (2), diallyl disulfide (3) and diallyl trisulfide (3) obtained from the corresponding standards (A) and from the garlic samples (B).



Supplementary Figure 4. Evolution of the relative concentration (percentage of the total peak areas) of furfural, 2-acetylfuran,5-methylfuranone, 3-penten-2-one, benzaldehyde and benzeneacetaldehyde in black garlic during the heating time.

CAPÍTULO IX

Establishing compositional differences between fresh and fermented garlic by a metabolomics approach based on LC–QTOF MS/MS analysis

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Establishing compositional differences between fresh and fermented garlic by a metabolomics approach based on LC-QTOF MS/MS analysis

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Establishing compositional differences between fresh and fermented garlic by a metabolomics approach based on LC-QTOF MS/MS analysis

María Molina-Calle, Verónica Sánchez de Medina, Feliciano Priego-Capote*, María D. Luque de Castro*

ABSTRACT

Despite black garlic is gaining popularity in the cuisine few studies have been devoted to in-depth profiling characterization of this product from fresh garlic. In this research polar extracts from fresh and black garlic of three garlic varieties cultivated in the South of Spain were characterized using liquid chromatography with tandem mass spectrometry in high resolution mode with a QTOF analyzer. Ninety-eight compounds were tentatively identified and classified in groups as a function of the given family: amino acids and derivatives, organosulfur compounds, saccharides and derivatives, lipids and derivatives, and others. The composition of the three garlic varieties was compared and the differences among them evaluated. Then, fresh and black garlic were compared to determine compounds exclusive of each one and also the compounds significantly different in concentration. The fermentation process changes considerably the composition of the amino acids family in garlic followed by organosulfur compounds.

Keywords Fresh garlic; Black garlic; LC−QTOF; Tentative identification; Polar extracts; Fermentation.

1. Introduction

Garlic (*Allium sativum*) is widely used over the world as seasoning ingredient, mainly in Asia, Africa, and Europe. Spain has become one of the major producers of garlic in the European Union, with a production of 173 million of tones of garlic in 2013 (FAOSTAT, 2013). Several studies have focused on the health beneficial properties of garlic to humans, such as the protective effect against cardiovascular diseases (Corzo-Martínez, Corzo, & Villamiel, 2007). Also cancer preventive properties have been attributed to garlic. Thus, Jin et al. (2013) associated garlic intake with a protective effect against lung cancer, and the study of Huang et al. (2011) demonstrated the antiproliferative effect of diallyl disulfide on colon cancer cells.

Garlic composition has been widely studied and reviewed, with special emphasis on organosulfur compounds, to which most of the beneficial properties of garlic are attributed. These compounds, mainly thiosulfinates and volatile compounds, and the used analytical methods for their identification and quantification were reviewed by Lanzotti (2006). As example, alkyl and alkenyl thiosulfinates from garlic homogenates were quantified using reversed-phase liquid chromatography (RP-LC) coupled to UV detection (Lawson, Wang, & Hughes, 1991; Lawson, Wood, & Hughes, 1991). Also, LC and gas chromatography (GC) coupled to mass spectrometry (LC–MS and GC−MS, respectively) and NMR have been used for analysis of organosulfur compounds from garlic and onion (Block, Naganathan, Putman, & Zhao, 1993). On the other hand, few studies have focused on other minor compounds present in garlic; as that of phenolic compounds and flavonoids, quantified in different Italian garlic varieties (Fratianni et al., 2016).

Nowadays, a garlic derivative that is gaining popularity is fermented garlic, also known as black garlic due to its dark color. Black garlic production is based on thermal processing, without additives, of fresh garlic for 1–3 months at 60–80

ºC under controlled humidity conditions. Under these conditions a number of chemical reactions, such as Maillard and enzymatic reactions, change the color of garlic from white to black, with also drastic transformation of both composition and flavor (Kim, Nam, Rico, & Kang, 2012).

The growing popularity of black garlic has promoted research on this product, showing that some characteristics, such as antioxidant capacity, are even higher in black than in fresh garlic. The majority of published studies have been devoted to the total content of phenolic compounds and flavonoids and/or the response against radical-scavenging tests (Choi, Cha, & Lee, 2014; J. S. Kim, Kang, & Gweon, 2013; Toledano Medina, Pérez-Aparicio, Moreno-Rojas, & Merinas-Amo, 2016; Wang, Liu, Yang, & Zhang, 2015). Other properties ascribed to black garlic are antiallergy activity (Kim, Nam, Rico, & Kang, 2012) and protection against diabetes effects (Jung et al., 2011). Some research has focused on the changes during the fermentation process of a given compound or family. This is the case with evaluation of the content of S-allyl-L-cysteine in garlic after subjection to low and high temperatures (Bae, Cho, Won, Lee, & Park, 2014). Also, the concentration of tetrahydro-β-carboline derivatives produced during garlic fermentation has been matter of study (Sato, Kohno, & Niwano, 2006). Nevertheless, one of the most comprehensive studies about composition of black garlic was carried out by Montaño, Casado, De Castro, Sánchez, & Rejano (2004), who quantified amino acids and some vitamins in fresh and black garlic; while Liang et al. (2015) differentiated fresh and black garlic using Nuclear Magnetic Resonance (NMR), obtaining a global profile of each sample, but with scant identification of individual compounds.

Looking for both an in depth characterization of the polar fraction of fresh garlic and the study of the changes produced during fermentation, the objectives of the present research were: (i) to tentatively characterize the polar extracts from fresh and black garlic; (ii) to distinguish global differences on composition among

different varieties of garlic; (iii) to qualitatively evaluate the compositional differences between fresh and black garlic and to determine the significant changes of the relative concentration of given compounds; and (iv) to evaluate the response of the three different garlic varieties to fermentation.

2. Materials and methods

2.1. Samples, reagents and standards

Fresh and black garlic from three varieties, *Purple* (var. Rocambole), *White* (var. Porcelain) and *Chinese* (var. Turban) were collected in 2014 at optimum ripening stage and provided by La Abuela Carmen (Montalbán, Córdoba, Spain). The bulbs of each variety were stored at −20 ^{o}C until use. Production of black garlic from each variety for our study involved division of fresh bulbs into cloves, peeling of the cloves and fermentation under high humidity and temperature within 60–80 ^{o}C in a closed chamber for 5 weeks.

Deionized water (18 MΩ·cm) was obtained by a Milli-Q water purification system from Millipore (Bedfore, MA, USA). Methanol was from Scharlab (Barcelona, Spain). L-Alanine, L-proline, L-valine, L-isoleucine, L-leucine, L-asparagine, L-aspartic acid, L-glutamine, L-lysine, L-glutamic acid, L-phenylalanine, L-tryptophan and L-arginine standards were from Sigma–Aldrich (St. Louis, USA). A multistandard solution with a concentration of 2 μg/mL of each standard in methanol was prepared and analyzed to confirm the presence of the target amino acids.

2.2. Apparatus and instruments

A vortex shaker IKA MS3 digital (Staufen, Germany) was used for the extraction procedure. An Agilent 1200 LC system consisting of a binary pump, a vacuum degasser, an autosampler, and a thermostated column compartment and coupled to an Agilent 6540 UHD Accurate-MS QTOF hybrid mass spectrometer

(Santa Clara, CA, USA) equipped with a dual electrospray (ESI) source was used to identify the compounds detected in the extracts. The analytical column was a Mediterranea Sea C18 analytical column (5 µm particle size, 15 × 0.46 cm) from Teknokroma (Barcelona, Spain).

2.3. Extraction procedure

Approximately 100 g of garlic bulbs were separated into cloves and cut into small pieces with a homogenous size. 0.5 g of each sample was placed in a 12-mL vial with 10 mL of a 50:50 (v/v) methanol–water mixture for the extraction of polar and mid-polar compounds. The vial was shaken in a vortex shaker for 30 min, and the obtained extract was filtered by a 0.20 µm filter and stored at –20 ºC until analysis.

2.4. LC−QTOF MS/MS analysis

The extracts were analyzed by LC–QTOF MS/MS in high resolution mode. The mobile phases were deionized water with 0.1% of formic acid (A), and methanol also with 0.1% of formic acid (B). The constant flow rate was 0.7 mL/min and the gradient was as follows: start at 5% B; change from 5% to 10% B in 5 min; from 10% to 100% B in 22.5 min and constant at 100% B for 2.5 min. Ten µL of sample of each sample vial located at the autosampler thermostated at 5 ºC was injected. The parameters of the dual electrospray ionization (ESI) source and mass spectrometer, operating in negative and positive ionization modes, were as follows: capillary, skimmer and Q1 voltage were set at 3500, 65 and 130 V, respectively; N_2 nebulizer gas was flowed at 40 psi; N_2 drying gas flow rate and temperature were 12 L/min and 325 ºC; and octopole radiofrequency voltage was set at 750 V. Two values of collision energy, 20 and 40 eV, were tested to combine the MS/MS data at two fragmentation levels. The MS and MS/MS range were from *m/z* 30 to 1700 and the data were collected at one spectrum per second in the mode of extended dynamic range. The auto MS/MS mode was configured with 2 maximum precursors per cycle and an exclusion window of 1 min after two

consecutive selections of the same precursor. To assure the desired mass accuracy of the recorded ions, continuous internal calibration was performed during analyses by the signals at *m/z* 131.0509 (protonated purine) and *m/z* 922.0098 [protonated hexakis (1H, 1H, 3H-tetrafluoropropoxy)phosphazine or HP-921] in positive ionization mode; and *m/z* 112.9856 (trifluoroacetic acid anion) and *m/z* 1033.9881 (HP-921) in negative ionization mode.

2.5. Data treatment

MassHunter Workstation software (version B 07.00 Qualitative Analysis, Agilent Technologies, Santa Clara, CA, USA) was used to extract potential molecular features (MFs) from all the data sets. The extraction algorithm considered all ions exceeding 1500 counts with a single charge. The isotopic distribution for inclusion of MFs should be defined by two or more ions (with a peak spacing tolerance of 0.0025 *m/z* and 10.0 ppm in mass accuracy). Protonated ions and adducts (+Na, +K) in positive ionization mode and deprotonated ion and chlorinated adduct (+Cl) in negative ionization mode, as well as neutral loss by dehydratation were included to identify molecular features (MFs) corresponding to the same potential metabolite. The raw data were filtered with a minimum counts level of 2500 for samples analyzed in the negative ionization mode and 3000 in the positive mode. The resulting MFs were tentatively identified by searching MS and MS/MS information on different databases such as METLIN (http://metlin.scripps.edu/), Food Database (http://foodb.ca/), MassBank (http://www.massbank.jp/) and MetFrag (http://msbi.ipb-halle.de/MetFrag/). An accuracy error limit of 5 ppm was set for identification.

In the next step Profinder (version B 6.00) from MassHunter Workstation software was used for alignment of the samples. A library with the identified compounds was created and used to consider only the peaks corresponding to these compounds. The alignment was carried out as a function of retention time and *m/z* value across the data matrix using a tolerance window of 0.2 min and 10

ppm mass accuracy. Finally, the resulting data matrix was exported into the Mass Profile Professional (MPP) software package (version 2.0, Agilent Technologies, Santa Clara, CA, USA) for statistical analysis.

3. Results and discussion

3.1. Tentative identification of compounds in fresh garlic

Taking into account the lack of research dealing with identification of compounds extracted from garlic, a preliminary study was focused on this task with the characterization of extracts from fresh garlic. Polar extracts from a pool of the three fresh garlic varieties were analyzed by LC−QTOF MS/MS as described in the Materials and methods section. The tentative identification of extracted MFs led to a total of 82 compounds with an accuracy error below 5 ppm both in MS and MS/MS data. The compounds tentatively identified using the existing databases were classified in families and grouped by related families, as can be seen in Table 1, which also lists the main parameters that support their identification. Supplementary Fig. 1 shows the comparison of MS/MS spectra of standards and amino acids detected and confirmed in fresh garlic.

The widest group of compounds is that including amino acids and derivatives, with a total of twenty-seven compounds, which were identified in positive ionization mode by generating the $[M+H]^+$, $[M+H-H_2O]^+$ and $[M+2H]^{2+}$ precursor ions. Eleven free amino acids were identified in fresh garlic, among which five are considered essential for humans (they are marked with an asterisk in Table 1). Also, two peptides and ten amino acid derivatives were identified in this group, with special emphasis on argininic acid due to the high intensity signal at which it was detected.

One other group identified in the samples was that of organosulfur compounds, characteristic of *Allium* species, including garlic. These compounds

were tentatively identified by formation of $[M+H]^+$ and $[M+NH_4]^+$ precursor ions in the positive ionization mode. Organosulfur compounds were classified in four main families: L-cysteine derivatives, sulfoxides, thiosulfinates and sulfur volatiles. On the basis of the review from Rose, Whiteman, Moore, & Zhu (2005) and the work from Yoshimoto et al., (2015) a metabolic pathway for the synthesis of these compounds in garlic is proposed in Fig. 1. The first step is the formation of γ-glutamyl-S-2-carboxypropyl-L-cysteine by condensation of L-cysteine and glutathione with subsequent addition of methylacrylate to the thiol group of the L-cysteine moiety. γ-Glutamyl-S-2-carboxypropyl-L-cysteine is the precursor of the γ-glutamyl-S-(alk(en)yl)-L-cysteine group, also detected in the extracts. In fact, the three main components of this group, the methyl, propenyl and allyl derivatives, were tentatively identified in the samples. These compounds lead to the S-(alk(en)yl)-L-cysteine via deglutamylation or the γ-glutamyl-S-(alk(en)yl)-L-cysteine sulfoxides via S-oxygenation. In the first case, two isomers were tentatively identified in the samples, S-allyl-L-cysteine and S-(1-propenyl)-L-cysteine; while in the second group the three main compounds γ-glutamyl-S-allyl-, γ-glutamyl-S-(1-propenyl)- and γ-glutamyl-S-methyl-L-cysteine sulfoxides were detected in fresh garlic. Both the S-(alk(en)yl)-L-cysteine and γ-glutamyl-S-(alk(en)yl)-L-cysteine sulfoxide families lead to the formation of sulfoxides by S-oxygenation and deglutamylation, respectively. Members of this family such as S-allyl-L-cysteine sulfoxide (alliin) and S-methyl-L-cysteine sulfoxide (methiin) were tentatively detected in the samples. As reviewed Lanzotti (2006), sulfoxides are broken under the enzymatic action of allinase and generate intermediate sulfenic acids that condensate to yield thiosulfinates. In this case, only diallyl thiosulfinate (allicin) and S-propyl-1-propanesulfinothioate were detected in garlic samples. The first compound has been described in the literature as the main responsible for the beneficial properties of garlic. Borlinghaus et al. reviewed the properties ascribed to allicin and highlighted its antimicrobial and antioxidant activities at a physiological level; its ability to stimulate immune cells activity and

anti-inflammatory effects, as well as its controversial relationship with cancer (Borlinghaus, Albrecht, Gruhlke, Nwachukwu, & Slusarenko, 2014). Thiosulfinates are very unstable compounds; thus, they are rapidly degraded giving place to the large variety of sulfur volatiles present in garlic. In our study, two of them, diallyl disulfide and diphenyl disulphide, were tentatively identified in fresh garlic.

Other main group of compounds tentatively identified in garlic was formed by saccharides and derivatives. These compounds were detected in both positive and negative ionization modes. A total of 18 components of this group were identified, classified in three families: saccharides, aminosaccharides and saccharide derivatives. On the other hand, 9 lipids and derivatives were identified in fresh garlic in positive ionization mode, excluding glycerophosphocholine, which was identified in the negative mode. Finally, 21 compounds belonging to different families were tentatively identified in fresh garlic (especial attention deserve ammonium salts such as choline, betaine and carnitine, and guanidine derivatives such as guanidino butanal and 4-guanidinobutanoic acid).

3.2. Comparison among fresh garlic varieties

A matrix was created with the compounds identified in each fresh garlic variety —*White*, *Purple* and *Chinese*— and exported to Mass Profile Professional software for statistical treatment. A heat map was created to evaluate the global concentration differences of the tentatively identified compounds among the three varieties. Supplementary Fig. 2 shows the heat map, in which the identified compounds are grouped in families. In overall terms, *Purple* and *White* garlic varieties showed a high similarity as compared to *Chinese* variety. This result was confirmed by evaluation of the significant compounds detected by their fold change ratio after applying an unpaired ANOVA. This information is included in Supplementary Table 1.

Table 1. Tentatively identified compounds in the polar extracts from fresh (F) and black (B) garlic and the main parameters that support identification.

Group	Family	Compound	Formula	Mass (Da)	RT (min)	*m/z*	Adduct	Fragments	Sample
Amino acids and derivatives (30)	Amino acids	Alanine	$C_3H_7NO_2$	89.0480	2.48	90.0550	$[M+H]^+$	44.0495, 55.9336	B
	Amino acids	Proline	$C_5H_9NO_2$	115.0640	2.82	116.0706	$[M+H]^+$	70.0648	F, B
	Amino acids	Valine*	$C_5H_{11}NO_2$	117.0790	2.65	118.0863	$[M+H]^+$	56.0545, 72.0810	F, B
	Amino acids	Isoleucine/Leucine*	$C_6H_{13}NO_2$	131.0950	6.18	132.1019	$[M+H]^+$	44.0408, 69.0596, 86.0952	F, B
	Amino acids	Asparagine	$C_4H_8N_2O_3$	132.0530	2.42	133.0608	$[M+H]^+$	46.0283, 74.0239, 87.0554	F, B
	Amino acids	Aspartic acid	$C_4H_7NO_4$	133.0400	2.52	134.0449	$[M+H]^+$	43.0187,70.0372, 72.0241	F, B
	Amino acids	Glutamine	$C_5H_{10}N_2O_3$	146.0700	2.63	147.0765	$[M+H]^+$	41.0390, 56.0504, 84.0452	F, B
	Amino acids	Lysine*	$C_6H_{14}N_2O_2$	146.1050	1.98	147.1128	$[M+H]^+$	67.0542, 84.0804, 101.1073	F, B
	Amino acids	Glutamic acid	$C_5H_9NO_4$	147.0510	2.59	148.0604	$[M+H]^+$	56.0490, 84.0444, 130.0488	F, B
	Amino acids	Phenylalanine*	$C_9H_{11}NO_2$	165.0790	12.31	166.0862	$[M+H]^+$	93.0704, 103.0545, 120.0806	F, B
	Amino acids	Tryptophan*	$C_{11}H_{12}N_2O_2$	174.1110	18.84	175.1189	$[M+H]^+$	146.0598, 159.0917, 170.0586	F
	Amino acids	Arginine	$C_6H_{14}N_4O_2$	103.0630	2.10	104.0711	$[M+H]^+$	60.0564, 70.0659, 116.0709	F, B
	Amino acid derivatives	Aminobutyric acid	$C_4H_9NO_2$	128.0580	2.28	129.0661	$[M+H]^+$	60.0809, 69.0334, 87.0385	F, B
	Amino acid derivatives	2-Amino-4-cyano-butanoic acid	$C_5H_8N_2O_2$	129.0790	2.63	130.0863	$[M+H]^+$	44.9933, 83.0596	F, B
	Amino acid derivatives	Pipecolic acid	$C_6H_{11}NO_2$	163.0470	1.98	164.0559	$[M+H]^+$	56.0501, 84.0813	F, B
	Amino acid derivatives	4-Hydroxy-glutamate	$C_6H_{13}NO_2$	175.0950	2.80	176.1032	$[M+H]^+$	84.0445, 130.0486	B
	Amino acid derivatives	Citrulline	$C_6H_{13}N_3O_3$	175.0950	2.61	176.1030	$[M+H]^+$	70.0657, 113.0710	F, B
	Amino acid derivatives	Argininic acid	$C_6H_{13}N_3O_3$	187.0480	2.10	186.0378	$[M+H]^+$	70.0649, 82.0660, 113.0723	F, B
	Amino acid derivatives	4-Hydroxy-2,3,4,5-tetrahydrodipicolinate	$C_7H_9NO_5$	188.1270	6.26	189.1354	$[M-H]^-$	142.0477	B
	Amino acid derivatives	NG-methyl-arginine	$C_7H_{16}N_4O_2$	320.1120	2.23	321.1297	$[M+H]^+$	70.0654, 74.0713, 116.0698	F, B

Cont. Table 1

	Amino acid derivatives	Isomugineic acid	$C_{12}H_{20}N_2O_8$	336.1640	2.96	337.1719	$[M+H]^+$	165.0654, 207.0764, 243.0973	F, B
	Amino acid derivatives	N2-Fructopyranosylarginine	$C_{12}H_{24}N_4O_7$	380.0910	2.15	381.1076	$[M+H]^+$	101.0220, 131.0798, 173.1009	B
	Amino acid derivatives	S-(N-Hydroxy-N-methylcarbamoyl)-glutathione	$C_{12}H_{20}N_4O_8S$	168.0670	3.07	169.0760	$[M+H]^+$	58.5757, 200.8759, 239.2361	F, B
	Amino acid derivatives	Tyramine	$C_8H_{11}NO$	204.0900	12.30	205.0972	$[M+H-H2O]^+$	77.0380, 94.0410, 103.0541	F, B
	Amino acid derivatives	Oxoproline	$C_5H_7NO_3$	258.0850	5.91	128.0324	$[M+H]^+$	44.0496, 56.0495, 84.0446	F, B
	Amino acid derivatives	Norharman	$C_{11}H_8N_2$	273.1280	20.63	274.1399	$[M+H]^+$	89.0392, 115.0545, 141.0576	B
	Peptides	Cys His	$C_9H_{14}N_4O_3S$	278.0890	5.95	277.0817	$[M+2H]^{2+}$	54.0327, 68.9945, 82.0268	B
	Peptides	Ala Pro Ser	$C_{11}H_{19}N_3O_5$	295.1260	3.03	293.1108	$[M+H]^+$	70.0635, 141.0983, 203.0991	B
	Peptides	N- ɣ -Glutamyl-methionine	$C_{10}H_{18}N_2O_5S$	137.0830	16.71	120.0803	$[M-H]^-$	47.2783, 82.3514, 162.0575	F, B
	Peptides	Glutamylphenylalanine	$C_{14}H_{19}N_2O_5$	129.0430	21.42	130.0498	$[M-H]^-$	128.0324, 147.0416, 164.0683	F, B
Lipids and derivatives (9)	Fatty acids and derivatives	Nonanedioic acid	$C_9H_{16}O_4$	229.2400	27.72	230.2475	$[M-H]^-$	97.0658, 125.0968	F, B
	Fatty acids and derivatives	Palmitoleic acid derivative	$C_{21}H_{41}NO_4$	134.0220	31.16	133.0115	$[M+H]^+$	57.0699, 255.2307	F, B
	Fatty acids and derivatives	Oleic acid derivative	$C_{23}H_{47}NO_4$	188.1050	32.16	187.0977	$[M+H]^+$	57.0699, 71.0855, 283.2630	F, B
	Fatty acids and derivatives	Gondoic acid derivative	$C_{25}H_{51}NO_4$	373.3190	32.94	374.3268	$[M+H]^+$	57.0699, 311.2940	F, B
	Glucosylceramides	GlcCer(d16:2/22:0(2OH))	$C_{44}H_{83}NO_9$	401.3510	27.82	402.3585	$[M+H+Na]^{2+}$	–	F, B
	Glycerides	MG(0:0/20:1/0:0)	$C_{23}H_{44}O_4$	429.3820	32.16	430.3893	$[M+NH4]^+$	283.2634	F, B
	Glycerides	DG(18:1/16:0/0:0)	$C_{25}H_{48}O_4$	769.6070	32.94	396.8014	$[M+NH4]^+$	311.2945	F, B
	Glycerophospholipids	Glycerophosphocholine	$C_8H_{21}NO_6P$	384.3240	2.59	402.3507	$[M-H]^-$	78.9566, 168.0401, 242.0776	F, B
	Sphingolipids and derivatives	C16 Sphinganine	$C_{16}H_{35}NO_2$	258.1070	29.25	302.0980	$[M+H]^+$	55.0557, 88.0753, 106.0868	F, B

Cont. Table 1

Organosulfur compounds (20)	L-cysteine derivatives	ɣ-Glutamyl-S-methyl-L-cysteine	$C_9H_{16}N_2O_5S$	273.2670	7.11	274.2750	$[M+H]^+$	84.0442, 119.0162	F, B
	L-cysteine derivatives	ɣ-Glutamyl-S-(1-propenyl)-L-cysteine	$C_{11}H_{18}N_2O_5S$	264.0780	20.14	265.0855	$[M+H]^+$	73.0096, 75.0250, 86.0224	F, B
	L-cysteine derivatives	ɣ -Glutamyl-S-allyl-L-cysteine	$C_{11}H_{18}N_2O_5S$	290.0940	18.31	291.1013	$[M+H]^+$	73.0093, 86.0224	F, B
	L-cysteine derivatives	ɣ -Glutamyl-S-allylthio-L-cysteine	$C_{11}H_{18}N_2O_5S_2$	290.0940	23.03	291.1013	$[M+H]^+$	69.0693, 249.0553, 288.9763	F, B
	L-cysteine derivatives	S-Allyl-L-cysteine	$C_6H_{11}NO_2S$	322.0660	6.76	323.0732	$[M+H]^+$	41.0389, 44.9796, 73.0105	F, B
	L-cysteine derivatives	S-(1-Propenyl)-L-cysteine	$C_6H_{11}NO_2S$	161.0490	9.00	162.0583	$[M+H]^+$	41.0389, 44.9796, 73.0105	F, B
	L-cysteine derivatives	S-allylmercapto-L-cysteine	$C_6H_{11}NO_2S_2$	193.0230	15.80	194.0304	$[M+H]^+$	91.0528, 117.9364, 135.0434	F, B
	Sulfoxides	ɣ -Glutamyl-S-allyl-L-cysteine sulfoxide	$C_{11}H_{18}N_2O_6S$	306.0890	5.12	307.0962	$[M+H]^+$	56.0498, 73.0107, 84.0446	F
	Sulfoxides	ɣ -Glutamyl-S-methyl-L-cysteine sulfoxide	$C_9H_{16}N_2O_6S$	280.0730	2.92	281.0807	$[M+H]^+$	84.0448, 88.0395, 130.0486	F, B
	Sulfoxides	ɣ -Glutamyl-S-(1-propenyl)-L-cysteine sulfoxide	$C_{11}H_{18}N_2O_6S$	306.0880	5.20	307.0962	$[M+H]^+$	56.0498, 73.0107, 84.0446	F, B
	Sulfoxides	Methyl-L-cysteine sulfoxide (Methiin)	$C_4H_9NO_3S$	146.0350	2.59	147.0297	$[M+H]^+$	44.9784, 47.9653, 62.9887	F, B
	Sulfoxides	Methionine sulfoxide	$C_5H_{11}NO_3S$	151.0300	2.66	152.0374	$[M+H]^+$	56.0490, 74.0228, 102.0537	F, B
	Sulfoxides	Allyl-L-cysteine sulfoxide (Alliin)	$C_6H_{11}NO_3S$	160.0200	3.19	178.0533	$[M+H]^+$	74.0249, 133.0506, 137.0116	F, B
	Sulfoxides	Cycloalliin	$C_6H_{11}NO_3S$	161.0490	3.61	162.0583	$[M+H]^+$	75.0312, 88.0387, 120.9537	F, B
	Thiosulfinates	Diallyl thiosulfinate (Allicin)	$C_6H_{10}OS_2$	162.0170	26.13	163.0243	$[M+H]^+$	94.0614, 109.0089, 133.0500	F
	Thiosulfinates	S-propyl 1-propanesulfinothioate	$C_6H_{14}OS_2$	165.0430	2.75	166.0534	$[M-H]^-$	56.9961, 59.0120, 75.0068	F, B
	Sulfur volatiles	Diallyl disulfide	$C_6H_{10}S_2$	166.0490	6.42	165.0412	$[M+H]^+$	74.0103, 75.0114	F, B
	Sulfur volatiles	Diphenyl disulfide	$C_{12}H_{10}S_2$	174.1120	2.71	173.1011	$[M+H]^+$	80.1816, 131.4861, 181.6235	F, B
	Others	Hexanethioic acid S-propyl ester	$C_9H_{18}OS$	177.0460	2.12	178.0536	$[M-H]^-$	56.4950, 85.0727, 131.8795	F, B
	Others	Thiacremonone	$C_6H_8O_3S$	177.0460	2.83	178.0536	$[M+NH4]^+$	74.0237, 88.0397, 102.0000	F, B

Cont. Table 1

Saccharides and derivatives (18)	Saccharides	Myo Inositol	$C_6H_{12}O_6$	218.0190	2.72	219.0269	$[M-H]^-$	59.0121, 71.0116, 85.0225	F, B
	Saccharides	Dulcitol	$C_6H_{14}O_6$	162.1260	2.68	163.1329	$[M-H]^-$	71.0117, 89.0219, 101.0207	F, B
	Saccharides	Gluconic acid	$C_6H_{12}O_7$	126.0310	2.82	127.0390	$[M-H]^-$	59.0150, 75.0095, 129.0202	F, B
	Saccharides	Fructose 6-phosphate	$C_6H_{13}O_9P$	137.0480	3.49	138.0554	$[M+H]^+$	81.0342, 109.0283	B
	Saccharides	Myo-inositol 1,4-bisphosphate	$C_6H_{14}O_{12}P_2$	103.1000	3.53	104.1070	$[M-H]^-$	78.9567, 158.9220, 241.0076	F, B
	Saccharides	Disaccharide phosphate	$C_{13}H_{23}O_{14}P$	118.0890	3.10	119.0869	$[M-H]^-$	78.9592, 96.9700, 255.2428	B
	Saccharides	Trisaccharide	$C_{18}H_{32}O_{16}$	161.1060	3.08	162.1126	$[M-H]^-$	89.0243, 179.0555, 221.0668	F, B
	Saccharides	Disaccharide	$C_{12}H_{22}O_{11}$	188.1520	3.06	189.2593	$[M-H]^-$	59.0156, 89.0259, 221.0692	F, B
	Saccharides	Glucose 1-phosphate	$C_6H_{13}O_9P$	427.3580	3.34	428.3736	$[M+H]^+$	120.9655, 147.0027, 185.0433	F, B
	Aminosaccharides	Glucosamine	$C_6H_{13}NO_5$	142.0270	2.71	141.0197	$[M+H]^+$	60.0461, 72.0462, 84.0459	F, B
	Aminosaccharides	N-Acetyl-D-fucosamine	$C_8H_{15}NO_5$	394.0880	2.59	395.0953	$[M-H]^-$	68.0485, 88.0384, 130.0479	B
	Aminosaccharides	Glycolyl-D-mannosamine/N-Glycolyl-D-glucosamine	$C_8H_{15}NO_7$	228.1200	2.49	229.1298	$[M+H]^+$	61.0391, 85.0183, 176.0799	B
	Aminosaccharides	Muramic acid	$C_9H_{17}NO_7$	129.0900	2.61	130.0974	$[M+H]^+$	72.0453, 126.0545, 144.0645	B
	Saccharide derivatives	Threonic acid	$C_4H_8O_5$	145.0850	2.83	146.0928	$[M-H]^-$	44.9972, 59.0119, 75.0065	F, B
	Saccharide derivatives	Sarmentosin epoxide	$C_{11}H_{17}NO_8$	146.0230	5.67	129.0178	$[M-H]^-$	112.9829, 128.0326, 191.0165	B
	Saccharide derivatives	Glyceryl laminarabinoside	$C_{15}H_{26}O_{14}$	232.1160	3.06	233.1248	$[M-H]^-$	75.0001, 105.0593, 106.0202	F, B
	Saccharide derivatives	Bis-D-fructose 2',1-2,1'-dianhydride	$C_{12}H_{20}O_{10}$	267.0970	3.10	268.1043	$[M-H]^-$	161.0481, 269.2116	F, B
	Saccharide derivatives	Fructose derivative	$C_6H_8O_4$	283.0920	2.72	284.0993	$[M+H]^+$	45.0330, 69.0335, 82.0637	F, B

Cont. Table 1

Others (21)	Aldehydes	5-Hydroxymethylfurfural	$C_6H_6O_3$	393.1200	14.39	392.1127	$[M+H]^+$	43.0157, 53.0372, 81.0234	B
	Alkaloids and derivatives	Dihydromacarpine	$C_{22}H_{19}NO_6$	192.0270	16.50	191.0163	$[M+H]^+$	113.0437,	F, B
	Alkaloids and derivatives	Trigonelline	$C_7H_7NO_2$	370.1120	2.83	371.1193	$[M+H]^+$	65.0391, 78.0341, 94.0655	F, B
	Ammonium salts	Choline	$C_5H_{13}NO$	136.0380	2.20	135.0302	$[M+H]^+$	44.0494, 45.0334, 58.0653	F, B
	Ammonium salts	Betaine	$C_5H_{12}NO_2$	430.1390	3.01	429.1317	$[M+H]^+$	58.0660, 59.0740	F
	Ammonium salts	Carnitine	$C_7H_{15}NO_3$	179.0790	2.29	180.0870	$[M+H]^+$	43.0189, 60.0816, 103. 0394	F, B
	Ammonium salts	Trimethyllysine	$C_9H_{20}N_2O_2$	180.0640	2.04	179.0528	$[M+H]^+$	70.0658, 98.0607, 113.9077	F, B
	Ammonium salts	Stearoylcarnitine	$C_{25}H_{49}NO_4$	182.0790	32.36	181.0720	$[M+H]^+$	60.0443, 366.4082, 396.4199	F, B
	Fatty alcohols	1,3,7-Octatrienol	$C_8H_{18}O_3$	196.0570	26.53	195.0523	$[M+H]^+$	56.9413, 80.9730, 98.9836	F, B
	Furoic acid and derivatives	5-Hydroxymethyl-2-furoic acid	$C_6H_6O_4$	205.0960	16.16	250.0939	$[M-H]^-$	51.0243, 113.7958	B
	Glycosilamines	5'-Butyrylphosphouridine	$C_{13}H_{19}N_2O_{10}P$	237.0840	3.06	238.0926	$[M+H]^+$	96.9673, 78.9937, 223.6438	F, B
	Guanidine derivatives	Deoxyguanidinoproclavaminic acid	$C_9H_{16}N_4O_3$	251.1010	2.41	252.1038	$[M+H]^+$	44.0418, 86.0733, 116.0721	B
	Guanidine derivatives	Guanidino butanal	$C_5H_{11}N_3O$	260.0290	2.45	261.0370	$[M+H]^+$	74.3823, 84.0448, 93.3566	F, B
	Guanidine derivatives	4-Guanidinobutanoic acid	$C_5H_{11}N_3O_2$	260.0300	3.04	261.0371	$[M+H]^+$	45.0348, 86.0606, 87.0445	F, B
	Lactones and derivatives	Dehydro-D-arabinono-1,4-lactone	$C_5H_6O_5$	291.0960	6.42	290.0852	$[M+H-H2O]^+$	56.9418, 60.9865, 68.9962	F, B
	Opines	Acetopine	$C_8H_{16}N_4O_4$	324.1060	2.50	359.0754	$[M+H]^+$	60.0554, 84.0806, 128.0706	B
	Purine and derivatives	Adenosine	$C_{10}H_{13}N_5O_4$	339.9960	10.17	338.9857	$[M+H]^+$	136.0614	F, B
	Purine and derivatives	Guanosine	$C_{10}H_{13}N_5O_5$	342.1160	12.91	341.1087	$[M+H]^+$	135.0285, 152.0560	F
	Carboxylic acids and derivatives	Malic acid	$C_4H_6O_5$	422.0830	3.98	421.0754	$[M-H]^-$	71.0117, 72.9913, 115.0012	F, B
	Carboxylic acids and derivatives	Citric acid	$C_6H_8O_7$	504.1690	6.50	503.1617	$[M-H]^-$	85.0275, 87.0067, 111.0066	F, B
	Flavonoids	Styraxin/Uralenol	$C_{20}H_{18}O_7$	144.0420	3.72	145.0499	$[M-H]^-$	73.0552, 86.0483, 98.1824	B

The asterisk marks the essential amino acids.

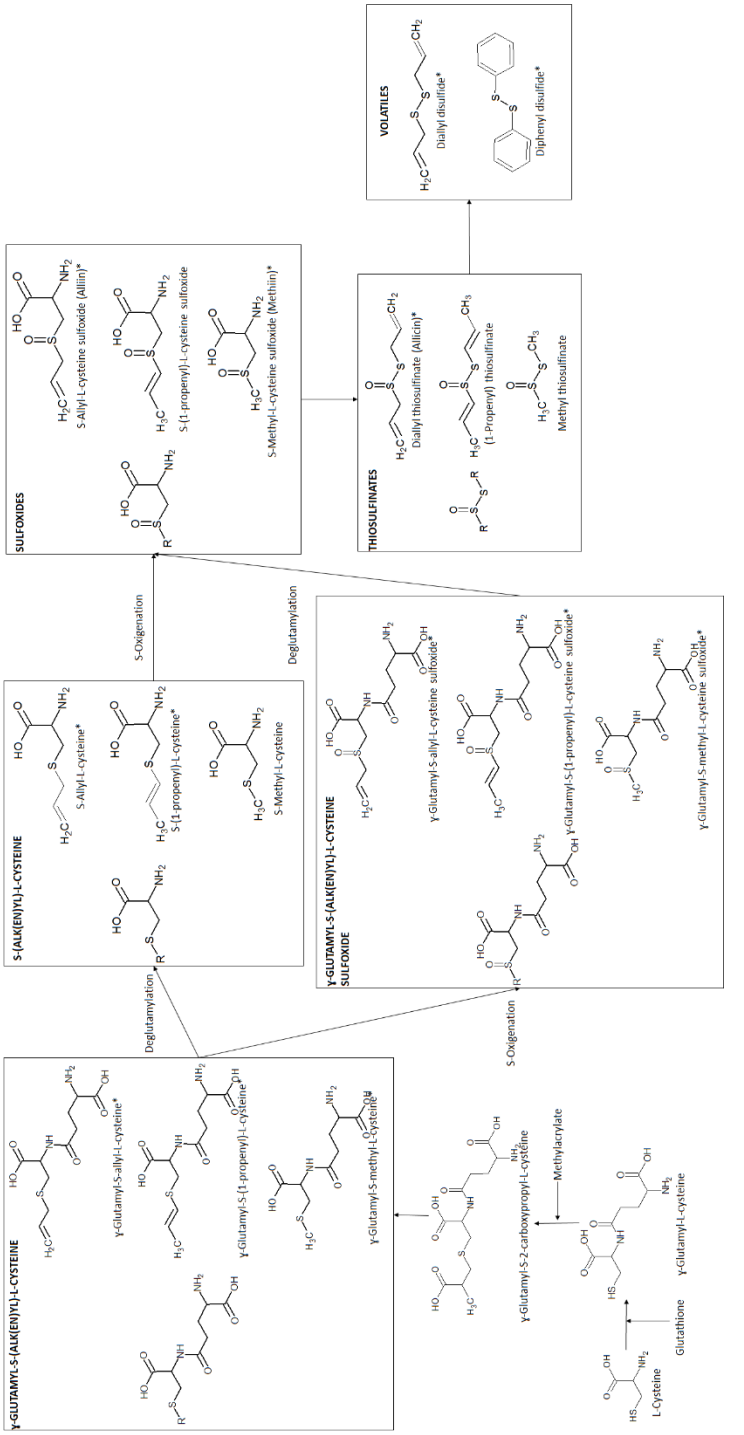

Figure 1. Metabolic pathways of the main organosulfur compounds found in garlic according to the literature. The tentatively identified compounds are marked with an asterisk.

The main differences between pairs of varieties were detected in amino acids and derivatives, in organosulfur compounds and in saccharides and derivatives. Thus, the *Purple* and *White* varieties reported highest levels of significant amino acids such as valine, asparagine, lysine or arginine than the *Chinese* variety, except for tryptophan, which was clearly at higher level in the *Chinese* garlic. Tryptophan is considered essential for humans and it is the precursor of serotonin, a neurotrasmitter related to happiness feeling (Schaechter & Wurtman, 1990), vitamin B_3 (Ikeda et al., 1963) and auxins, a class of plant hormones involved in growth processes (Palme & Nagy, 2008). Also, tryptophan is the precursor of tetrahydro-β-carboline derivatives such as norharman, which are characteristic of black garlic, as discussed later. Additionally, the *Purple* garlic reported, in general, higher levels of amino acids and derivatives than *White* garlic, except for 2-amino-4-cyano-butanoic acid.

Similarly, the organosulfur compounds were detected at lower concentration in *Chinese* garlic as compared to *White* and *Purple* varieties, except for diphenyl disulfide that was more concentrated in *Chinese* garlic than in *Purple* garlic. In this case, the differences between *White* and *Purple* varieties were not so clear and some organosulfur compounds were more concentrated in *Purple* garlic such as glutamyl-S-methyl-cysteine, glutamyl-S-allyl-cysteine and methiin; while glutamyl-S-1-propenyl-cysteine, glutamyl-S-allylthio-cysteine and glutamyl-S-allyl-cysteine sulfoxide were more concentrated in *White* garlic.

Some particular differences were also found in the composition of saccharides and derivatives, which were more concentrated in the *White* variety followed by the *Chinese* garlic.

3.3. Fresh versus black garlic

A new data set was created with the results obtained from fresh and black garlic for comparison between both products by statistical analysis. For this

purpose, the samples were divided into two groups, fresh and black garlic (independently of the variety), and an entity list was created with the compounds present in all the samples in each group, resulting in a matrix formed by 12 samples × 98 compounds (93 compounds for black garlic and 80 for fresh garlic). This filtering step allowed removing the compounds reporting variability among varieties. The entity lists for fresh and black garlic were compared by a Venn diagram that revealed 75 compounds detected in the two types of garlic. On the other hand, 18 and 5 compounds were exclusively detected in black and fresh garlic, respectively (see Fig. 2). Among compounds exclusive of fresh garlic, and concerning the amino acids and derivatives group, tryptophan was the unique detected only in fresh garlic. Allicin, one of the most characteristic compounds in garlic, together with γ-glutamyl-S-allyl-L-cysteine sulfoxide, were only present in fresh garlic, as did betaine and guanosine. Alanine, one of the main amino acids, was not detected as such in fresh garlic. Also, two peptides and four amino acid derivatives were exclusively detected in black garlic. One of these derivatives was norharman, formed by condensation of tryptophan with pyruvic acid; thus suggesting that tryptophan reacts during garlic fermentation to form norharman, which concentrates in black garlic.

Figure 2. Venn diagram from the compounds identified in the polar extract from black garlic *versus* fresh garlic and the exclusive compounds of each set of samples.

In dealing with saccharides and derivatives, three saccharides, two aminosaccharides and a derivative were only detected in black garlic. A furfural derivative, 5-hydromethylfurfural, was exclusively found in black garlic since this compound is generated in the Maillard reaction of sugars degradation. This process occurs during the fermentation of the garlic (Molina-Calle, Priego-Capote, & Luque de Castro, 2016).

Concerning the 75 compounds common to fresh and black garlic, a Volcano test (unpaired *t*-test combined to a fold change analysis) was applied to determine the compounds with concentration significantly different between the two types of garlic. The cut-off *p*-value and fold change ratio were set at 0.01 and 2.0, respectively, and a Bonferroni FWER test correction was applied. A total of 37 compounds were present at significantly different concentration in fresh and black garlic, as shows Table 2. The samples that provided the highest concentration of each compound are also pointed out: +F and +B correspond to a higher concentration in fresh and black garlic, respectively.

As can be globally seen, amino acids and derivatives is the group with major number of significant different compounds. Within this group, 16 compounds provided significant differences in concentration between fresh and black garlic that, together with 8 compounds exclusive of each type of garlic, yield an 80% of the tentatively identified amino acids and derivatives affected by fermentation. Concerning amino acids, lysine and arginine were present in fresh garlic in a significantly higher concentration than in black garlic, while the opposite happened for glutamine, phenylalanine and aspartic acid. The trend for amino acid derivatives depended on the compound. Some of the tentatively identified amino acids and derivatives can be related among them by different pathways of synthesis and degradation of the main amino acids. This relationship, combined with the different concentration between fresh and black garlic, provides some evidences about the pathways of amino acids affected by garlic fermentation.

Fragments of synthesis and degradation pathways of arginine, alanine, aspartic acid and lysine are shown in Fig. 3. As can be seen in the first pathway, arginine, argininic acid and amino butyric acid were significantly more concentrated in fresh garlic, while 4-guanidinobutanoic acid and citrulline resulted to be in a significant higher concentration in black garlic; acetopine and deoxyguanidino-proclavaminic acid were found only in this garlic product. This behavior leads to consider citrulline, acetopine, deoxyguanidinoproclavaminic acid and 4-guanidinobutanoic acid as degradation products of arginine formed during garlic fermentation. Concerning the second pathway, aspartic acid and glutamine were found in a significant higher concentration in black garlic and, as previously commented, alanine and 4-hydroxy-2,3,4,5-tetrahydrodipicolate were exclusive of this garlic; while fresh garlic provided a significant higher concentration of lysine and pipecolic acid. This suggests that the transformation step from 4-hydroxy-2,3,4,5-tetrahydrodipicolate to lysine is favored in fresh garlic, and not in black garlic, producing the accumulation of 4-hydroxy-2,3,4,5-tetrahydrodipicolate in this case.

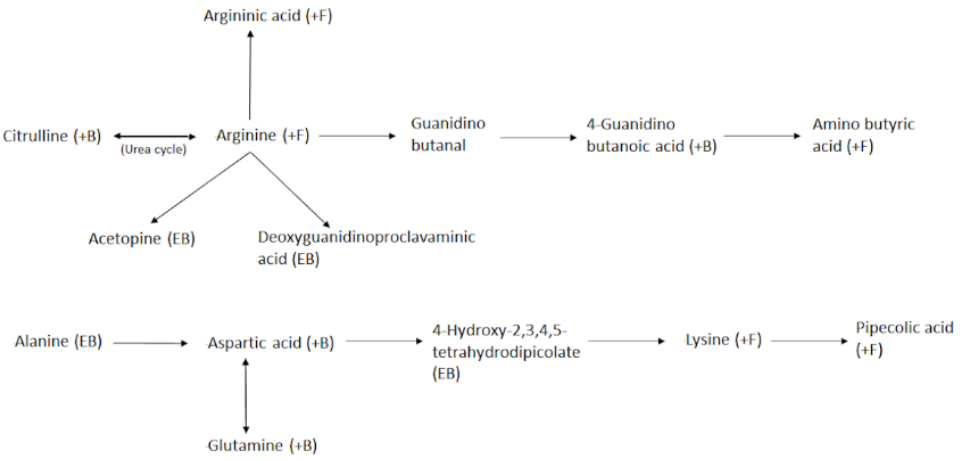

Figure 3. Summary of the pathways for synthesis and degradation of arginine, alanine, aspartic acid and lysine. All the compounds included in the scheme were tentatively identified. The compounds found at higher concentration in fresh or black garlic are marked with (+F) and (+B), respectively, while (EB) corresponds to compounds exclusively detected in black garlic.

Table 2. Compounds resulting from the Volcano test with *p*-value and fold change ratios and the type of garlic in which they are significantly more concentrated: +F for fresh garlic and +B for black garlic.

Group	Compound	*p*-Value	FC-Value	Sample
Amino acids and derivatives	Lysine	0.005438189	2.6	+F
	Arginine	1.18E-04	4.9	+F
	Glutamine	2.84E-07	3.7	+B
	Phenylalanine	2.61E-04	3.4	+B
	Aspartic acid	5.88E-05	5.2	+B
	2-Amino-4-cyano-butanoic acid	2.50E-09	7.4	+B
	Glutamylphenylalanine	3.81E-08	4.7	+F
	Aminobutyric acid	0.00134759	2.4	+F
	S-(N-Hydroxy-N-methylcarbamoyl)glutathione	2.68E-05	3.5	+F
	Argininic acid	0.002325986	5.2	+F
	Pipecolic acid	0.004778366	2.7	+F
	Citrulline	3.27E-05	8.6	+B
	Tyramine	0.001314551	3.1	+B
	4-Guanidinobutanoic acid	7.86E-14	38.3	+B
	Isomugineic acid	2.29E-04	37.4	+B
	Oxoproline	8.81E-17	88.1	+B
Organosulfur compounds	γ-Glutamyl-S-(1-propenyl)-L-cysteine	1.83E-07	297.1	+F
	γ -Glutamyl-S-allyl-L-cysteine	0.002279875	23.8	+F
	S-Allylmercapto-L-cysteine	1.14E-04	3.3	+F
	Allyl-L-cysteine sulfoxide (Alliin)	0.00313919	30.2	+F
	Cycloalliin	4.06E-13	145.7	+F
	Methyl-L-cysteine sulfoxide (Methiin)	1.14E-10	30.3	+F
	Methionine sulfoxide	1.39E-09	9.6	+B
	S-Propyl 1-propanesulfinothioate	1.89E-19	11.7	+B
	Diphenyl disulfide	4.22E-08	3.7	+B
	Thiacremonone	3.60E-04	2.8	+B
Saccharides and derivatives	Trisaccharide	8.80E-11	11.7	+F
	Glucose-1-phosphate	1.51E-11	32.7	+B
	Gluconic acid	3.81E-14	22.6	+B
	Myo-inositol-1,4-bisphosphate	0.004618582	6.4	+B
	Glucosamine	2.46E-13	6.4	+B
	Glyceryl laminarabinoside	1.98E-14	9.4	+B
	Fructose derivative	5.90E-13	7.6	+B
	Bis-D-fructose 2',1-2,1'-dianhydride	2.24E-10	179.2	+B
	Threonic acid	1.59E-18	52.2	+B
Others	Carnitine	6.26E-07	3.0	+B
	5'-Butyrylphosphouridine	4.68E-05	7.9	+B

Concerning organosulfur compounds, 10 of those detected in fresh and black garlic were in significantly different concentration between them. It should be highlighted that most of the significantly different compounds between fresh and black garlic are present at higher concentration in the former. The two most

remarkable compounds were γ-glutamyl-S-(1-propenyl)-L-cysteine and cycloalliin, whose FC-values were considerably high: they are present in a much greater concentration in fresh garlic than in black garlic. The opposite behavior showed methionine sulfoxide, S-propyl 1-propanesulfinothioate, diphenyl disulfide and thiacremonone, which resulted to be in a significantly higher concentration in black garlic. Thiacremonone is described in the literature as an organosulfur product from treatment of fresh garlic with high pressure and high temperature, and to which recent studies attributed healthy properties such as antiobesity effect (Ban et al., 2012), decreased proliferation of lung cancer (Jo et al., 2014) and anti-inflamatory effect (Ban et al., 2009). When the differences in concentration of organosulfur compounds are evaluated, their behavior suggests that either fermentation decreases the formation of intermediates compounds (cysteine derivatives, sulfoxides and thiosulfinates) or fermentation favors the subsequent transformation of the intermediates to the final products, which are organosulfur volatiles (see Fig. 1).

Concerning the saccharides and derivatives group, 9 compounds resulted with a significantly different concentration between fresh and black garlic; all of them more concentrated in black garlic, except a compound tentatively identified as a trisaccharide, with a higher concentration in fresh garlic. The 8 compounds more concentrated in black garlic —in addition to the six saccharides and derivatives exclusive of black garlic— showed that the tentatively identified compounds within this group are characteristic of the fermented garlic, which can result from the rupture of long-chain polysaccharides and/or from the degradation of the main saccharides present in fresh garlic by the Maillard reaction, which is in agreement with the study from Zhang et al. (2015). Finally, two compounds that do not belong to any specific group are in a significantly higher concentration in black garlic: carnitine and 5'-butyrylphosphouridine. Several studies have reported that carnitine —three times more concentrated in

black garlic— decreases the amount of ammonia in the organism, which can decrease the side effects of hepatic diseases, especially in the case of hepatic encephalopathy (Examine, 2016).

3.4. Comparison of black garlic from different varieties

A Principal Component Analysis (PCA) was also carried out to evaluate the variability in composition of polar extracts obtained from the three fresh garlic varieties and their fermented products. The resulting PCA plot (Fig. 4) showed two main groups corresponding to fresh and black garlic products, which means that the main variability source is ascribed to the fermentation process.

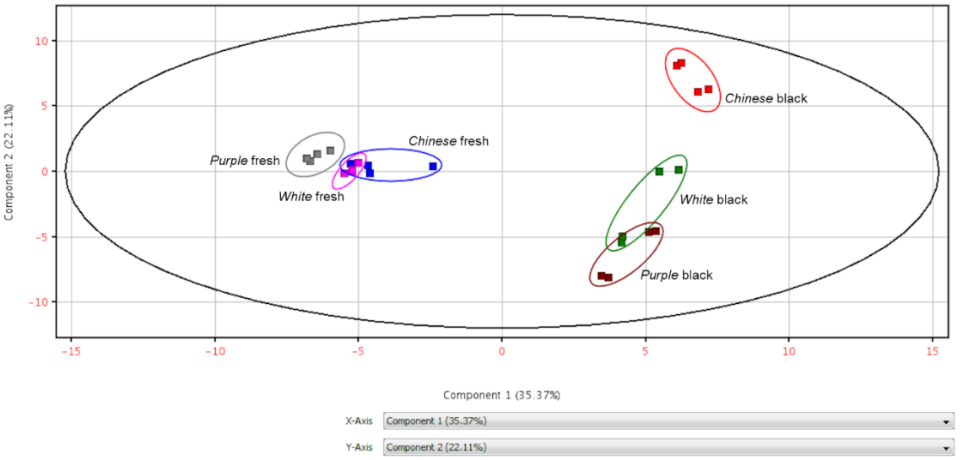

Figure 4. Principal Component Analysis of fresh and black garlic from the three tested varieties.

Comparing these two groups, it should be highlighted that a higher differentiation was observed in the black garlic products than in fresh varieties along the PC2. This behavior suggests that the variability in composition of fresh garlic is enhanced after the fermentation process.

An unpaired ANOVA test was applied to black garlic samples to determine the compounds that provided significant differences among them in terms of concentration. Supplementary Table 2 shows the compounds resulting from this

test and the obtained *p*-value and fold change ratio for the comparison of black garlic from the three varieties: *Purple*, *White* and *Chinese*. Thirteen compounds provided a significantly different concentration among the three samples, which included five amino acids and derivatives, five organosulfur compounds, two saccharides and derivatives and dihydromacarpine, classified as other compounds. Within amino acids and derivatives, asparagine provided a higher concentration in *Chinese* black garlic, followed by *White* black variety, as show the fold change values. This behavior is in contrast to that found when the fresh garlic varieties were compared since the *Purple* fresh garlic variety reported the highest concentration of this amino acid. This fact suggests that the fermentation process drastically affects the concentration of asparagines, but this change is strongly associated to the garlic variety. Supplementary Fig. 3 shows that asparagine practically disappears in black garlic from the *Purple* variety, while it increases the concentration in the *Chinese* variety due to fermentation.

Concerning organosulfur compounds, two of them, γ-glutamyl-S-methyl-L-cysteine and γ-glutamyl-S-(1-propenyl)-L-cysteine, resulted exclusive of black garlic from *White* and *Chinese* varieties, respectively. On the other hand, γ-glutamyl-S-allyl-L-cysteine was clearly more concentrated in *White* garlic, as suggest the fold change values. However, when compared to the fresh samples, a strong decrease in the concentration of this compound due to fermentation can be observed in all the varieties (see Supplementary Fig. 3). This demonstrates that the γ-glutamyl-S-(alk(en)yl)-L-cysteine group is the most affected by fermentation in the metabolic pathway of the organosulfur compounds (see Fig. 1).

Finally, two saccharides and derivatives, glucose-1-phosphate and bis-fructose 2',1-2,1'-dianhydride, resulted in a significant higher concentration in black garlic from *Purple* garlic, while dihydromacarpine was exclusive in the fermented product of the *White* variety.

4. Conclusions

In this study, comprehensive profiling of the polar extracts from three fresh garlic varieties and their fermented products (black garlic) was carried out using LC−QTOF MS/MS equipment. A total of 98 compounds were tentatively identified in polar extracts from the samples, and were classified in different families, grouped into amino acids and derivatives, organosulfur compounds, saccharides and derivatives, lipids and derivatives, and others. Firstly, the three varieties were compared by a heat map, where a high level of similarity was found between *White* and *Purple* garlic. The results showed that the main differences among the three fresh garlic varieties were due to the amino acids and derivatives, and organosulfur compounds.

Comparison between fresh and black garlic by a Venn diagram (involving all tentatively identified compounds), and a Volcano test (involving only common compounds) allowed determining exclusive compounds and compounds with a significantly different concentration in fresh and black garlic. The obtained information leads to the conclusion that amino acids and derivatives was the group more affected by fermentation, since some of them were present at significantly higher concentration in fresh garlic, while others showed the opposite behavior. Additionally, the results supported on the proposed synthesis pathway for organosulfur compounds revealed the behavior of these compounds when subjected to the fermentation conditions. Therefore, intermediates organosulfur compounds in this pathway are less concentrated in black garlic, thus either their formation is not favored or their transformation into the final products (volatiles) is enhanced. Finally, the individual study of fresh and black garlic of the three varieties showed that the concentration of some compounds allowed distinguishing the different behavior against fermentation of the three varieties under study.

Acknowledgements

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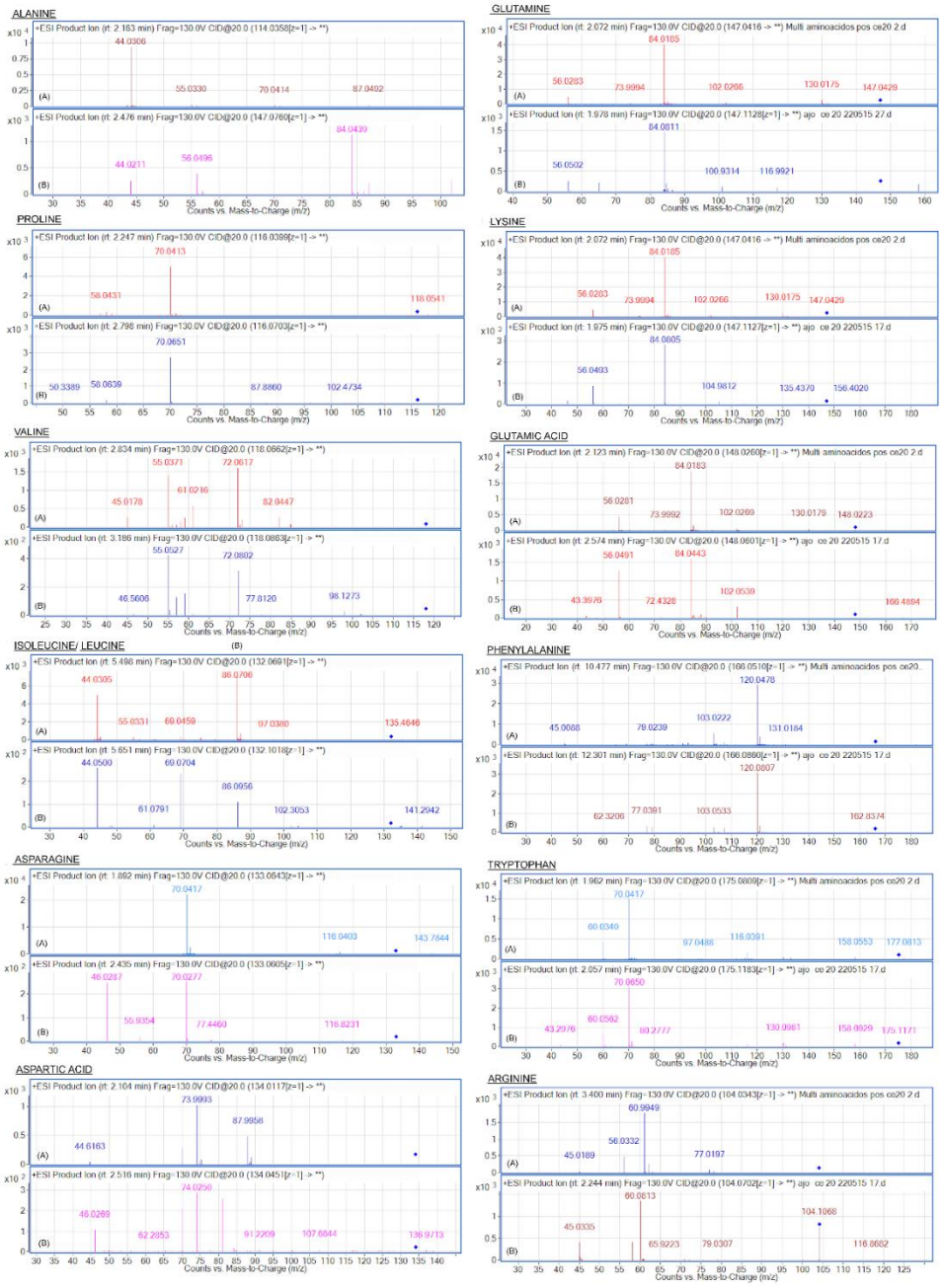
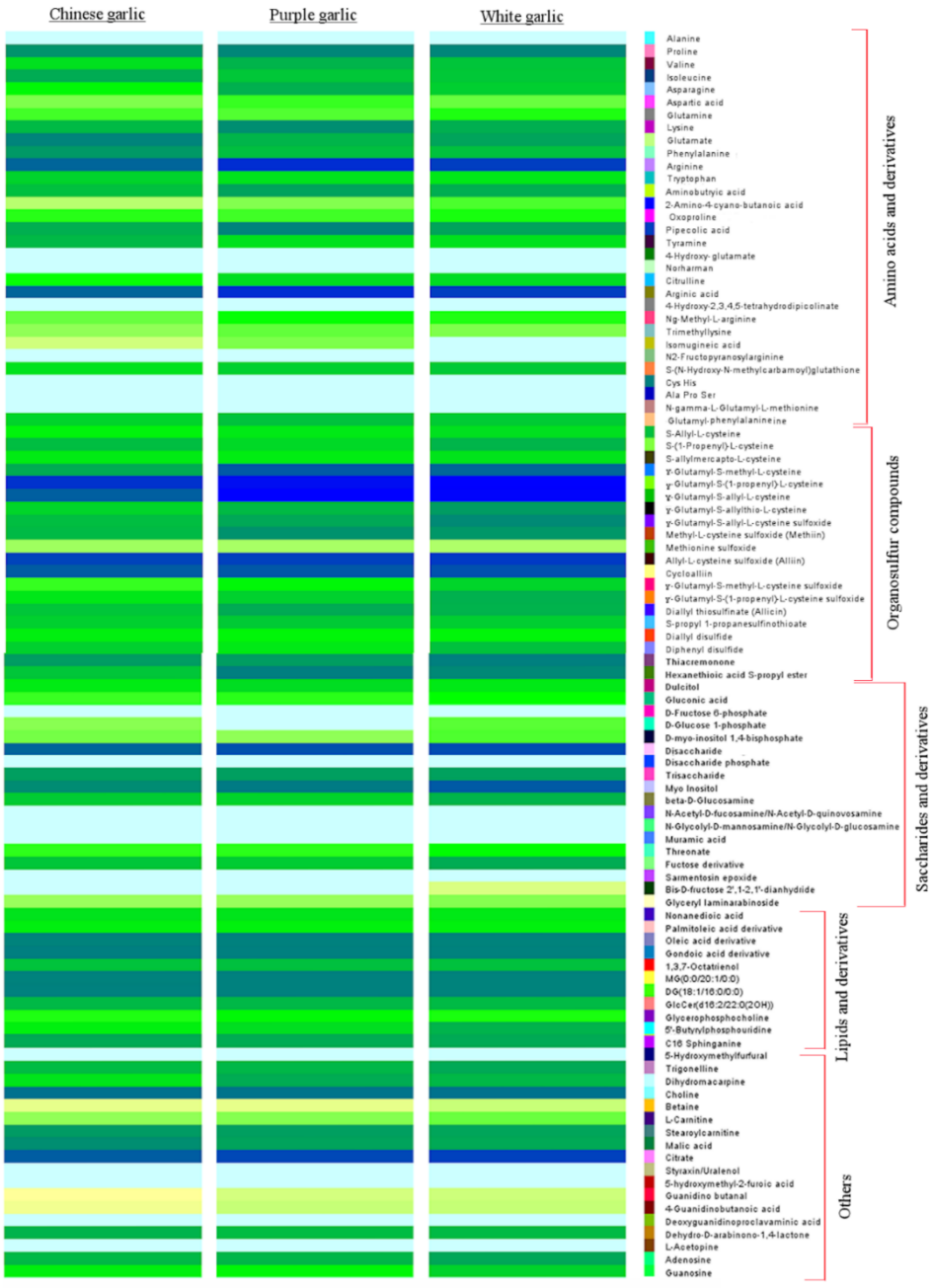
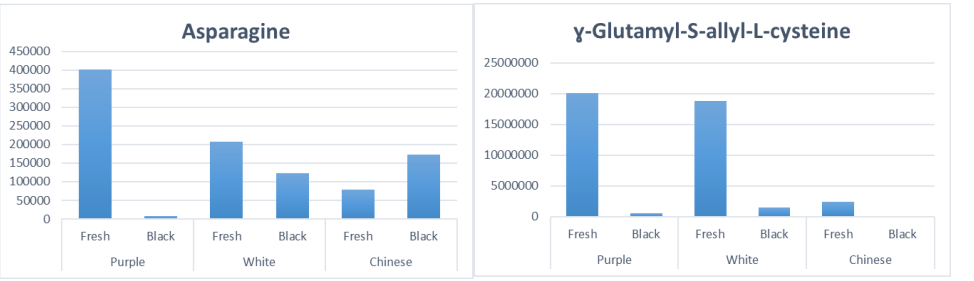
Supplementary information



Supplementary Figure 1. MS/MS spectra of the identified amino acids obtained by analysis of the corresponding standards (A) and the extract from the garlic samples (B).



Supplementary Figure 2. Heat map comparing the three varieties of fresh garlic under study (*Chinese*, *Purple* and *White*).



Supplementary Figure 3. Normalized quantitative response of asparagine and ɣ-glutamyl-S-allyl-L-cysteine reported by analysis of polar extracts from fresh and black garlic of the three varieties.

Supplementary Table 1 Compounds found at significantly different concentration in the three fresh garlic varieties. The *p*-value and fold change ratio are also listed.

Compound	*p*-Value	Chinese (C) *vs* Purple (P)		Chinese (C) *vs* White (W)		Purple (P) *vs* White (W)	
		FC-Value	Concentration	FC-Value	Concentration	FC-Value	Concentration
Valine	8.13×10^{-7}	2.6	+P	1.8	+W	1.4	+P
Asparagine	7.49×10^{-4}	5.1	+P	2.5	+W	2.0	+P
Lysine	3.28×10^{-4}	2.7	+P	1.3	+W	2.1	+P
Arginine	2.88×10^{-4}	3.5	+P	2.4	+W	1.5	+P
Tryptophan	0.0092	1.4	+C	1.6	+C	1.1	+P
Aminobutyric acid	0.0157	1.9	+P	1.5	+W	1.3	+P
2-Amino-4-cyano-butanoic acid	0.0044	1.7	+P	2.8	+W	1.7	+W
Pipecolic acid	5.63×10^{-4}	2.7	+P	1.3	+W	2.1	+P
Argininic acid	3.14×10^{-4}	3.5	+P	2.4	+W	1.5	+P
Isomugeneic acid	0	2.2	+P				
Glutamyl-phenylalanine	0.0148	1.5	+P	1.2	+W	1.2	+P
ɣ-Glutamyl-S-methyl-L-cysteine	4.26×10^{-10}	6.4	+P	4.8	+W	1.3	+P
ɣ -Glutamyl-S-(1-propenyl)-L-cysteine	5.72×10^{-5}	2.3	+P	3.0	+W	1.3	+W
ɣ -Glutamyl-S-allyl-L-cysteine	5.00×10^{-7}	8.4	+P	7.7	+W	1.1	+P
ɣ -Glutamyl-S-allylthio-L-cysteine	0.0389	1.8	+P	3.6	+W	2.0	+W
ɣ -Glutamyl-S-allyl-L-cysteine sulfoxide	0.0423	1.6	+P	3.2	+W	2.0	+W
Methyl-L-cysteine sulfoxide (Methiin)	0.0061	2.4	+P	2.2	+W	1.1	+P
Diphenyl disulfide	0.0056	1.7	+C	1.4	+W	2.4	+W
Glucose-1-phosphate	0			1.6	+W		
Myo-inositol-1,4-bisphosphate	0	1.3	+C	1.4	+W	1.9	+W
Glucosamine	4.72×10^{-4}	1.2	+C	1.7	+W	2.0	+W
Fructose derivative	4.48×10^{-4}	1.1	+C	1.8	+W	2.0	+W
Dihydromacarpine	0.0018	3.5	+P	2.7	+W	1.3	+P
Betaine	0	1.0	+P	1.4	+W	1.3	+W

Supplementary Table 2 Compounds found at significantly different concentration in the three black garlic varieties. The *p*-value and fold change ratio are also listed.

Compound	*p*-Value	Chinese (C) *vs* Purple (P)		Chinese (C) *vs* White (W)		Purple (P) *vs* White (W)	
		FC-Value	Concentration	FC-Value	Concentration	FC-Value	Concentration
Asparagine	0	23.9	+C	1.6	+C	15.293421	+W
Arginine	0.011176	2.4	+C	1.5	+W	3.6845753	+W
Aminobutryic acid	0.007223	2.7	+C	1.7	+C	1.6438723	+W
Argininic acid	0					3.6864316	+W
Isomugineic acid	0.000474	2.1	+C	3.6	+C	1.7049029	+P
ɣ-Glutamyl-S-methyl-L-cysteine	0	Exclusive of black garlic from *White* variety					
ɣ -Glutamyl-S-(1-propenyl)-L-cysteine	0	Exclusive of black garlic from *Chinese* variety					
ɣ -Glutamyl-S-allyl-L-cysteine	0.000121	28.4	+P	75.4	+W	2.650519	+W
ɣ -Glutamyl-S-allylthio-L-cysteine	0					2.46712	+W
Methyl-L-cysteine sulfoxide (Methiin)	0	1.9	+C	1.5	+C	1.2745736	+W
Glucose 1-phosphate	0.000732	1.8	+P	1.4	+W	1.2892869	+P
Bis-fructose 2',1-2,1'-dianhydride	0.024279	2.0	+P	1.2	+W	1.6818885	+P
Dihydromacarpine	0	Exclusive of black garlic from *White* variety					

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Untargeted analysis to monitor metabolic changes of garlic along fermentation by LC–QTOF MS/MS

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Untargeted analysis to monitor metabolic changes of garlic along fermentation by LC–QTOF MS/MS

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Untargeted analysis to monitor metabolic changes of garlic along fermentation by LC–QTOF MS/MS

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ABSTRACT

Black garlic is increasing its popularity in cuisine around the world; however, scant information exists on the composition of this processed product. In this study, polar compounds in fresh garlic and in samples taken at different times during the fermentation process to obtain black garlic have been characterized by liquid chromatography coupled to tandem mass spectrometry in high resolution mode. Ninety-five compounds (mainly amino acids and metabolites, organosulfur compounds, and saccharides and derivatives) were tentatively identified in all the analyzed samples and classified as a function of the family they belong to. Statistical analysis of the results allowed establishing that the major changes in garlic occur during the first days of fermentation, and they mainly affect to the three representative families. The main pathways involved in the synthesis of the compounds affected by fermentation, and their evolution during the fermentation process were studied.

Keywords: Black garlic; Fermentation; LC−QTOF MS/MS; Tentative identification; Metabolic pathway.

1. Introduction

Black garlic is a product obtained by fermentation of fresh garlic that is gaining popularity nowadays thanks to its properties, drastically different from those of fresh garlic. Black garlic has been widely used from years in the Asian cuisine but it is currently being expanded throughout Europe and America. This product is obtained without additives by subjecting fresh garlic to a thermal process for 1–3 months at 60–80 ^{o}C under controlled humidity conditions. Under these conditions, chemical reactions (such as Maillard and enzymatic reactions) take place thus promoting the color change from white to black and the drastic transformation of both composition and flavor resulting in an intense sweet taste [1].

Several studies have focused on the healthy properties of black garlic, with special emphasis on its anti-allergic activity [1], protection effect against diabetes [2], anti-hypertensive activity [3], and the increased function of immune cells after black garlic intake [4]. Ha et al. studied the effect of black garlic intake in rats and reported a decrease in the concentration of lipids, triglycerides and cholesterol in blood [5].

The growing popularity of black garlic together with its healthy properties have promoted research on the characteristics and composition of this product. Most of the research published in this context has been devoted to study a given parameter such as the total content of phenols and flavonoids, and/or the response to radical-scavenging tests [6–9]. Some research has been addressed to the changes of a given compound or family during the fermentation process. Bae et al. evaluated the content of S-allyl-L-cysteine in garlic after subjection to low and high temperatures [10], while Sato et al. studied the concentration of tetrahydro-β-carboline derivatives (including norharman) produced during garlic fermentation [11]. On the other hand, one of the most comprehensive studies about the composition of black garlic was carried out by Montaño et al., who quantified

amino acids and some vitamins in fresh and black garlic [12]. Also, Kim et al. quantified eleven compounds belonging to the organosulfur family in black garlic fermented with the help of different bacteria [13]. Finally, Liang et al. used NMR to monitor the compositional changes among fresh and fermented garlic during 5, 25 and 90 days, being able to determinate the evolution of 38 individual compounds. Within these compounds, 17 amino acids and derivatives were identified, while only 6 saccharides and 2 organosulfur compounds (allicin and cycloalliin) could be detected by NMR [14].

Taking into account the previous studies, the objectives of the present research were: (i) to tentatively identify the polar compounds present in fresh and black garlic and in samples taken at different times during fermentation; (ii) to evaluate the evolution of the overall composition during the process; (iii) to determine the evolution and behavior of individual compounds during fermentation; and (iv) to establish the effect of fermentation on some relevant biochemical pathways in garlic.

2. Materials and methods

2.1. Samples, reagents and standards

Bulbs from fresh garlic of the Purple variety (var. Rocambole) were divided into cloves and fermented under high humidity and temperature within 60–80 ^{o}C in a closed chamber for 36 days by La Abuela Carmen (Montalbán, Córdoba, Spain). Clove samples of fresh garlic and at six stages corresponding to 6, 12, 18, 24, 30 and 36 days of fermentation were taken, then stored at −20 ^{o}C until use.

Deionized water (18 MΩ·cm) was obtained by a Milli-Q water purification system from Millipore (Bedfore, MA, USA). Methanol was from Scharlab (Barcelona, Spain). L-Alanine, L-proline, L-valine, L-isoleucine, L-leucine, L-asparagine, L-aspartic acid, L-glutamine, L-lysine, L-glutamic acid, L-

phenylalanine, L-tryptophan and L-arginine standards were from Sigma–Aldrich (St. Louis, USA). A multistandard solution with a concentration of 2 μg/mL of each standard in methanol was prepared and analyzed to confirm the presence of these amino acids.

2.2. Apparatus and instruments

A vortex shaker IKA MS3 digital (Staufen, Germany) was used for extraction. An Agilent 1200 LC system consisting of a binary pump, a vacuum degasser, an autosampler, and a thermostated column compartment was coupled to an Agilent 6540 UHD Accurate-MS QTOF hybrid mass spectrometer (Santa Clara, CA, USA) equipped with a dual electrospray (ESI) source. The analytical column was a Mediterranea Sea C18 (5 μm particle size, 15 × 0.46 cm) from Teknokroma (Barcelona, Spain).

2.3. Extraction procedure

Cloves (ca. 100 g) were cut into small pieces with a homogeneous size, and 0.5 g of each sample was placed in a 12-mL vial with 10 mL of a 50:50 (v/v) methanol–water mixture as extractant. The vial was vortexed for 30 min, and the extract thus produced was filtered by a 0.20 μm filter and stored at –20 ^{o}C until analysis.

2.4. LC−QTOF MS/MS analysis

The extracts were analyzed by LC–QTOF MS/MS in high resolution mode. The mobile phases were deionized water with 0.1% of formic acid (A), and methanol also with 0.1% of formic acid (B). The constant flow rate was 0.7 mL/min and the gradient was as follows: start at 5% B; change from 5% to 10% B in 5 min; from 10% to 100% B in 22.5 min and constant at 100% B for 2.5 min. The injection volume was 10 μL from each sample vial located at the autosampler thermostated at 5 ^{o}C. The parameters of the dual electrospray ionization (ESI) source and mass spectrometer, operating in negative and positive ionization modes, were as

follows: capillary, skimmer and Q1 voltage were set at 3500, 65 and 130 V, respectively; N2 nebulizer gas flowed at 40 psi; N2 drying gas flow rate and temperature were 12 L/min and 325 ºC; and the octopole radiofrequency voltage was set at 750 V. Two values of collision energy, 20 and 40 eV, were tested to combine MS/MS data at two fragmentation levels. The MS and MS/MS range were from *m/z* 30 to 1700 and the data were collected at one spectrum/s in the extended dynamic range mode. The auto MS/MS mode was configured with 2 maximum precursors per cycle and an exclusion window of 1 min after two consecutive selections of the same precursor. To assure the desired mass accuracy of the recorded ions continuous internal calibration was performed during analyses by the signals at *m/z* 131.0509 (protonated purine) and *m/z* 922.0098 [protonated hexakis (1H, 1H, 3H-tetrafluoropropoxy)phosphazine or HP-921] in the positive ionization mode; and *m/z* 112.9856 (trifluoroacetic acid anion) and *m/z* 1033.9881 (HP-921) in the negative ionization mode.

2.5. Data treatment

MassHunter Workstation software (version B 07.00 Qualitative Analysis, Agilent Technologies, Santa Clara, CA, USA) was used to extract potential molecular features (MFs) from all the data sets. The extraction algorithm considered all ions exceeding 1500 counts with a single charge. The isotopic distribution for inclusion of molecular features (MFs) should be defined by two or more ions (with a peak spacing tolerance of 0.0025 *m/z* and 10.0 ppm in mass accuracy). Protonated ions and adducts (+Na, +K) in positive ionization mode and deprotonated ion and chlorinated adduct (+Cl) in negative ionization mode, as well as neutral loss by dehydratation were included to identify MFs corresponding to the same potential metabolite. The raw data were filtered with a minimum counts level of 2500 for samples analyzed in the negative ionization mode and 3000 in the positive mode. The resulting MFs were tentatively identified by searching MS and MS/MS information on different databases such as METLIN

(http://metlin.scripps.edu/), Food Database (http://foodb.ca/), MassBank (http://www.massbank.jp/) and MetFrag (http://msbi.ipb-halle.de/MetFrag/). An accuracy error limit of 5 ppm was set for identification.

In the next step Profinder (version B 6.00) from MassHunter Workstation software was used for samples alignment. A library with the identified compounds was created and used to consider only the peaks corresponding to these compounds. The alignment was carried out as a function of retention time and *m/z* value across the data matrix using a tolerance window of 0.2 min and 10 ppm mass accuracy. Finally, the resulting data matrix was exported into the Mass Profile Professional (MPP) software package (version 2.0, Agilent Technologies, Santa Clara, CA, USA) for statistical analysis.

3. Results and discussion

3.1. Tentative identification of compounds present in fresh and fermented garlic

A unique data set was generated after analysis of fresh garlic and fermented garlic at different times. Three aliquots from each sample were analyzed. The criteria for inclusion of molecular entities in the data set were that the feature should be detected in at least two samples and in all the aliquots from each sample. With these premises, the tentative identification of extracted MFs led to 95 compounds with an accuracy error below 5 ppm both in MS and MS/MS data. The compounds tentatively identified using the existing databases were classified in families and grouped by related families, as can be seen in Table 1, which also lists the main parameters that support identification. The presence of amino acids was confirmed by analysis of standards.

The widest group of identified compounds included amino acids and metabolites (thirty compounds), which were essentially detected in positive ionization mode by generation of the [M+H]+, [M+H−H2O]+ and [M+2H]2+

precursor ions. Eleven free amino acids, among which five are considered essential for humans (they are marked with an asterisk in Table 1); four peptides, and thirteen amino acid metabolites were identified in the garlic samples.

Organosulfur compounds (characteristic of Allium species) were tentatively identified in the samples by formation of [M+H]+ and [M+NH4]+ precursor ions. They were classified into four main families: L-cysteine derivatives, sulfoxides, thiosulfinates and sulfur volatiles. Within the tentatively identified compounds, S-allyl-L-cysteine sulfoxide (alliin) and diallyl thiosulfinate (allicin) should be highlighted since these compounds are described in the literature as the major organosulfur compounds found in fresh garlic. Many of the beneficial properties of garlic have been attributed to both compounds [15].

Other main group of compounds tentatively identified in garlic was that formed by saccharides and derivatives, which were detected in both positive and negative ionization modes. A total of 18 components of this group were identified. Additionally, 9 lipids and derivatives were identified in fresh garlic in positive ionization mode, excluding glycerophosphocholine that was identified in the negative mode. Finally, 15 compounds belonging to different families were tentatively identified in the extracts, among which carnitine, adenosine, guanidine, and malic and citric acids should be highlighted.

A preliminary result of the compositional changes occurring in garlic during the fermentation process is shown in Supplementary Fig. 1. The Venn diagram compares the number of MFs found in fresh garlic, black garlic, and an intermediate sample corresponding to garlic fermented for 18 days. As can be seen, 9 molecular features were exclusively detected in fresh garlic. However, a representative number of features was found in garlic samples subjected to fermentation. This diagram reveals the drastic compositional changes occurring when fresh garlic is fermented under controlled conditions.

Table 1. Tentatively identified compounds in the polar extracts from fresh and black garlic and the main parameters supporting identification.

Family	Compound	Formula	Mass (Da)	RT (min)	*m/z*	Adduct	Fragments
Amino acids and derivatives (32)							
Amino acids	Proline	$C_5H_9NO_2$	115.0640	2.82	116.0706	$[M+H]^+$	70.0648
Amino acids	Valine*	$C_5H_{11}NO_2$	117.0790	2.65	118.0863	$[M+H]^+$	56.0545, 72.0810
Amino acids	Isoleucine/Leucine*	$C_6H_{13}NO_2$	131.0950	6.18	132.1019	$[M+H]^+$	44.0408, 69.0596, 86.0952
Amino acids	Asparagine	$C_4H_8N_2O_3$	132.0530	2.42	133.0608	$[M+H]^+$	46.0283, 74.0239, 87.0554
Amino acids	Aspartic acid	$C_4H_7NO_4$	133.0400	2.52	134.0449	$[M+H]^+$	43.0187,70.0372, 72.0241
Amino acids	Glutamine	$C_5H_{10}N_2O_3$	146.0700	2.63	147.0765	$[M+H]^+$	41.0390, 56.0504, 84.0452
Amino acids	Lysine*	$C_6H_{14}N_2O_2$	146.1050	1.98	147.1128	$[M+H]^+$	67.0542, 84.0804, 101.1073
Amino acids	Glutamic acid	$C_5H_9NO_4$	147.0510	2.59	148.0604	$[M+H]^+$	56.0490, 84.0444, 130.0488
Amino acids	Phenylalanine*	$C_9H_{11}NO_2$	165.0790	12.31	166.0862	$[M+H]^+$	93.0704, 103.0545, 120.0806
Amino acids	Tryptophan*	$C_{11}H_{12}N_2O_2$	174.1110	18.84	175.1189	$[M+H]^+$	146.0598, 159.0917, 170.0586
Amino acids	Arginine	$C_6H_{14}N_4O_2$	103.0630	2.10	104.0711	$[M+H]^+$	60.0564, 70.0659, 116.0709
Amino acid metabolites	Aminobutyric acid	$C_4H_9NO_2$	128.0580	2.28	129.0661	$[M+H]^+$	60.0809, 69.0334, 87.0385
Amino acid metabolites	2-Amino-4-cyano-butanoic acid	$C_5H_8N_2O_2$	129.0790	2.63	130.0863	$[M+H]^+$	44.9933, 83.0596
Amino acid metabolites	Pipecolic acid	$C_6H_{11}NO_2$	163.0470	1.98	164.0559	$[M+H]^+$	56.0501, 84.0813
Amino acid metabolites	4-Hydroxy-glutamic acid	$C_6H_{13}NO_2$	175.0950	2.80	176.1032	$[M+H]^+$	84.0445, 130.0486
Amino acid metabolites	Citrulline	$C_6H_{13}N_3O_3$	175.0950	2.61	176.1030	$[M+H]^+$	70.0657, 113.0710
Amino acid metabolites	Argininic acid	$C_6H_{13}N_3O_3$	187.0480	2.10	186.0378	$[M+H]^+$	70.0649, 82.0660, 113.0723

Cont. Table 1

Amino acid metabolites	NG-methyl-arginine	$C_7H_{16}N_4O_2$	320.1120	2.23	321.1297	$[M+H]^+$	70.0654, 74.0713, 116.0698
Amino acid metabolites	4-Hydroxy-2,3,4,5-tetrahydrodipicolinate	$C_7H_9NO_5$	188.1270	6.26	189.1354	$[M-H]^-$	142.0477
Amino acid metabolites	Isomugineic acid	$C_{12}H_{20}N_2O_8$	336.1640	2.96	337.1719	$[M+H]^+$	165.0654, 207.0764, 243.0973
Amino acid metabolites	N2-Fructopyranosylarginine	$C_{12}H_{24}N_4O_7$	380.0910	2.15	381.1076	$[M+H]^+$	101.0220, 131.0798, 173.1009
Amino acid metabolites	S-(N-Hydroxy-N-methylcarbamoyl)glutathione	$C_{12}H_{20}N_4O_8S$	168.0670	3.07	169.0760	$[M+H]^+$	58.5757, 200.8759, 239.2361
Amino acid metabolites	Tyramine	$C_8H_{11}NO$	204.0900	12.30	205.0972	$[M+H-H2O]^+$	77.0380, 94.0410, 103.0541
Amino acid metabolites	Oxoproline	$C_5H_7NO_3$	258.0850	5.91	128.0324	$[M+H]^+$	44.0496, 56.0495, 84.0446
Amino acid metabolites	Deoxyguanidinoproclavaminic acid	$C_9H_{16}N_4O_3$	251.1010	2.41	252.1038	$[M+H]^+$	44.0418, 86.0733, 116.0721
Amino acid metabolites	4-Guanidinobutanoic acid	$C_5H_{11}N_3O_2$	260.0300	3.04	261.0371	$[M+H]^+$	45.0348, 86.0606, 87.0445
Amino acid metabolites	Trimethyllysine	$C_9H_{20}N_2O_2$	180.0640	2.04	179.0528	$[M+H]^+$	70.0658, 98.0607, 113.9077
Amino acid metabolites	Norharman	$C_{11}H_8N_2$	273.1280	20.63	274.1399	$[M+H]^+$	89.0392, 115.0545, 141.0576
Peptides	Cys His	$C_9H_{14}N_4O_3S$	278.0890	5.95	277.0817	$[M+2H]^{2+}$	54.0327, 68.9945, 82.0268
Peptides	Ala Pro Ser	$C_{11}H_{19}N_3O_5$	295.1260	3.03	293.1108	$[M+H]^+$	70.0635, 141.0983, 203.0991
Peptides	γ -Glutamyl-methionine	$C_{10}H_{18}N_2O_5S$	137.0830	16.71	120.0803	$[M-H]^-$	47.2783, 82.3514, 162.0575
Peptides	Glutamylphenylalanine	$C_{14}H_{19}N_2O_5$	129.0430	21.42	130.0498	$[M-H]^-$	128.0324, 147.0416, 164.0683
Lipids and derivatives (9)							
Fatty acids and derivatives	Nonanedioic acid	$C_9H_{16}O_4$	229.2400	27.72	230.2475	$[M-H]^-$	97.0658, 125.0968
Fatty acids and derivatives	Palmitoleic acid derivative	$C_{21}H_{41}NO_4$	134.0220	31.16	133.0115	$[M+H]^+$	57.0699, 255.2307

Cont. Table 1

Fatty acids and derivatives	Oleic acid derivative	$C_{23}H_{47}NO_4$	188.1050	32.16	187.0977	$[M+H]^+$	57.0699, 71.0855, 283.2630
Fatty acids and derivatives	Gondoic acid derivative	$C_{25}H_{51}NO_4$	373.3190	32.94	374.3268	$[M+H]^+$	57.0699, 311.2940
Glucosylceramides	**GlcCer(d16:2/22:0(2OH))**	**$C_{44}H_{83}NO_9$**	**401.3510**	**27.82**	**402.3585**	**$[M+H+Na]^{2+}$**	–
Glycerides	MG(0:0/20:1/0:0)	$C_{23}H_{44}O_4$	429.3820	32.16	430.3893	$[M+NH4]^+$	283.2634
Glycerides	DG(18:1/16:0/0:0)	$C_{25}H_{48}O_4$	769.6070	32.94	396.8014	$[M+NH4]^+$	311.2945
Glycerophospholipids	Glycerophosphocholine	$C_8H_{21}NO_6P$	384.3240	2.59	402.3507	$[M-H]^-$	78.9566, 168.0401, 242.0776
Sphingolipids and derivatives	C16 Sphinganine	$C_{16}H_{35}NO_2$	258.1070	29.25	302.0980	$[M+H]^+$	55.0557, 88.0753, 106.0868
Organosulfur compounds (20)							
L-cysteine derivatives	γ-Glutamyl-S-methyl-L-cysteine	$C_9H_{16}N_2O_5S$	273.2670	7.11	274.2750	$[M+H]^+$	84.0442, 119.0162
L-cysteine derivatives	γ-Glutamyl-S-(1-propenyl)-L-cysteine	$C_{11}H_{18}N_2O_5S$	264.0780	20.14	265.0855	$[M+H]^+$	73.0096, 75.0250, 86.0224
L-cysteine derivatives	γ -Glutamyl-S-allyl-L-cysteine	$C_{11}H_{18}N_2O_5S$	290.0940	18.31	291.1013	$[M+H]^+$	73.0093, 86.0224
L-cysteine derivatives	γ -Glutamyl-S-allylthio-L-cysteine	$C_{11}H_{18}N_2O_5S_2$	290.0940	23.03	291.1013	$[M+H]^+$	69.0693, 249.0553, 288.9763
L-cysteine derivatives	S-Allyl-L-cysteine	$C_6H_{11}NO_2S$	322.0660	6.76	323.0732	$[M+H]^+$	41.0389, 44.9796, 73.0105
L-cysteine derivatives	S-(1-Propenyl)-L-cysteine	$C_6H_{11}NO_2S$	161.0490	9.00	162.0583	$[M+H]^+$	41.0389, 44.9796, 73.0105
L-cysteine derivatives	S-Alylmercapto-L-cysteine	$C_6H_{11}NO_2S_2$	193.0230	15.80	194.0304	$[M+H]^+$	91.0528, 117.9364, 135.0434
Sulfoxides	γ -Glutamyl-S-allyl-L-cysteine sulfoxide	$C_{11}H_{18}N_2O_6S$	306.0890	5.12	307.0962	$[M+H]^+$	56.0498, 73.0107, 84.0446
Sulfoxides	γ -Glutamyl-S-methyl-L-cysteine sulfoxide	$C_9H_{16}N_2O_6S$	280.0730	2.92	281.0807	$[M+H]^+$	84.0448, 88.0395, 130.0486

Cont. Table 1

Sulfoxides	γ -Glutamyl-S-(1-propenyl)-L-cysteine sulfoxide	$C_{11}H_{18}N_2O_6S$	306.0880	5.20	307.0962	$[M+H]^+$	56.0498, 73.0107, 84.0446
Sulfoxides	Methyl-L-cysteine sulfoxide (Methiin)	$C_4H_9NO_3S$	146.0350	2.59	147.0297	$[M+H]^+$	44.9784, 47.9653, 62.9887
Sulfoxides	Methionine sulfoxide	$C_5H_{11}NO_3S$	151.0300	2.66	152.0374	$[M+H]^+$	56.0490, 74.0228, 102.0537
Sulfoxides	Allyl-L-cysteine sulfoxide (Alliin)	$C_6H_{11}NO_3S$	160.0200	3.19	178.0533	$[M+H]^+$	74.0249, 133.0506, 137.0116
Sulfoxides	Cycloalliin	$C_6H_{11}NO_3S$	161.0490	3.61	162.0583	$[M+H]^+$	75.0312, 88.0387, 120.9537
Thiosulfinates	Diallyl thiosulfinate (Allicin)	$C_6H_{10}OS_2$	162.0170	26.13	163.0243	$[M+H]^+$	94.0614, 109.0089, 133.0500
Thiosulfinates	S-Propyl 1-propanesulfinothioate	$C_6H_{14}OS_2$	165.0430	2.75	166.0534	$[M-H]^-$	56.9961, 59.0120, 75.0068
Sulfur volatiles	Diallyl disulfide	$C_6H_{10}S_2$	166.0490	6.42	165.0412	$[M+H]^+$	74.0103, 75.0114
Sulfur volatiles	Diphenyl disulfide	$C_{12}H_{10}S_2$	174.1120	2.71	173.1011	$[M+H]^+$	80.1816, 131.4861, 181.6235
Others	Hexanethioic acid S-propyl ester	$C_9H_{18}OS$	177.0460	2.12	178.0536	$[M-H]^-$	56.4950, 85.0727, 131.8795
Others	Thiacremonone	$C_6H_8O_3S$	177.0460	2.83	178.0536	$[M+NH4]^+$	74.0237, 88.0397, 102.0000
Saccharides and derivatives (18)							
Saccharides	Myo Inositol	$C_6H_{12}O_6$	218.0190	2.72	219.0269	$[M-H]^-$	59.0121, 71.0116, 85.0225
Saccharides	Dulcitol	$C_6H_{14}O_6$	162.1260	2.68	163.1329	$[M-H]^-$	71.0117, 89.0219, 101.0207
Saccharides	Gluconic acid	$C_6H_{12}O_7$	126.0310	2.82	127.0390	$[M-H]^-$	59.0150, 75.0095, 129.0202
Saccharides	Fructose 6-phosphate	$C_6H_{13}O_9P$	260.0297	3.49	138.0554	$[M+H]^+$	96.9702, 109.0283
Saccharides	Ribitol-5-phosphate	$C_5H_{13}O_8P$	232.1257	2.51	231.1134	$[M-H]^-$	78.9591, 96.9702, 231.1132
Saccharides	Myo-inositol 1,4-bisphosphate	$C_6H_{14}O_{12}P_2$	103.1000	3.53	104.1070	$[M-H]^-$	78.9567, 158.9220, 241.0076

Cont. Table 1

Saccharides	Disaccharide phosphate	$C_{13}H_{23}O_{14}P$	118.0890	3.10	119.0869	$[M-H]^-$	78.9592, 96.9700, 255.2428
Saccharides	Raffinose	$C_{18}H_{32}O_{16}$	504.1661	3.04	503.1649	$[M-H]^-$	89.0243, 179.0555, 221.0668
Saccharides	Maltose	$C_{12}H_{22}O_{11}$	342.1130	2.99	341.1009	$[M-H]^-$	59.0156, 89.0259, 221.0692
Saccharides	Glucose-1-phosphate	$C_6H_{13}O_9P$	427.3580	3.34	428.3736	$[M+H]^+$	120.9655, 147.0027, 185.0433
Saccharides	Glucose-6-phosphate	$C_6H_{13}O_9P$	427.3580	2.75	428.3734	$[M+H]^+$	120.9655, 147.0027, 185.0433
Aminosaccharides	Glucosamine	$C_6H_{13}NO_5$	142.0270	2.71	141.0197	$[M+H]^+$	60.0461, 72.0462, 84.0459
Aminosaccharides	N-Glycolyl-D-glucosamine	$C_8H_{15}NO_7$	228.1200	2.49	229.1298	$[M+H]^+$	61.0391, 85.0183, 176.0799
Aminosaccharides	Muramic acid	$C_9H_{17}NO_7$	129.0900	2.61	130.0974	$[M+H]^+$	72.0453, 126.0545, 144.0645
Saccharide derivatives	Threonic acid	$C_4H_8O_5$	145.0850	2.83	146.0928	$[M-H]^-$	44.9972, 59.0119, 75.0065
Saccharide derivatives	Sarmentosin epoxide	$C_{11}H_{17}NO_8$	146.0230	5.67	129.0178	$[M-H]^-$	112.9829, 128.0326, 191.0165
Saccharide derivatives	Glyceryl laminarabinoside	$C_{15}H_{26}O_{14}$	232.1160	3.06	233.1248	$[M-H]^-$	75.0001, 105.0593, 106.0202
Saccharide derivatives	Bis-D-fructose 2',1-2,1'-dianhydride	$C_{12}H_{20}O_{10}$	267.0970	3.10	268.1043	$[M-H]^-$	161.0481, 269.2116
Others (15)							
Aldehydes	5-Hydroxymethylfurfural	$C_6H_6O_3$	393.1200	14.39	392.1127	$[M+H]^+$	43.0157, 53.0372, 81.0234
Alkaloids and derivatives	Dihydromacarpine	$C_{22}H_{19}NO_6$	192.0270	16.50	191.0163	$[M+H]^+$	113.0437,
Ammonium salts	Choline	$C_5H_{13}NO$	136.0380	2.20	135.0302	$[M+H]^+$	44.0494, 45.0334, 58.0653
Ammonium salts	Carnitine	$C_7H_{15}NO_3$	179.0790	2.29	180.0870	$[M+H]^+$	43.0189, 60.0816, 103. 0394

Cont. Table 1

Ammonium salts	Stearoylcarnitine	$C_{25}H_{49}NO_4$	182.0790	32.36	181.0720	$[M+H]^+$	60.0443, 366.4082, 396.4199
Fatty alcohols	1,3,7-Octatrienol	$C_8H_{18}O_3$	196.0570	26.53	195.0523	$[M+H]^+$	56.9413, 80.9730, 98.9836
Furoic acid and derivatives	5-Hydroxymethyl-2-furoic acid	$C_6H_6O_4$	205.0960	16.16	250.0939	$[M-H]^-$	51.0243, 113.7958
Glycosilamines	5'-Butyrylphosphouridine	$C_{13}H_{19}N_2O_{10}P$	237.0840	3.06	238.0926	$[M+H]^+$	96.9673, 78.9937, 223.6438
Lactones and derivatives	Dehydro-D-arabinono-1,4-lactone	$C_5H_6O_5$	291.0960	6.42	290.0852	$[M+H-H2O]^+$	56.9418, 60.9865, 68.9962
Opines	Acetopine	$C_8H_{16}N_4O_4$	324.1060	2.50	359.0754	$[M+H]^+$	60.0554, 84.0806, 128.0706
Purine and derivatives	Adenosine	$C_{10}H_{13}N_5O_4$	339.9960	10.17	338.9857	$[M+H]^+$	136.0614
Purine and derivatives	Guanosine	$C_{10}H_{13}N_5O_5$	342.1160	12.91	341.1087	$[M+H]^+$	135.0285, 152.0560
Carboxylic acids and derivatives	Malic acid	$C_4H_6O_5$	422.0830	3.98	421.0754	$[M-H]^-$	71.0117, 72.9913, 115.0012
Carboxylic acids and derivatives	Citric acid	$C_6H_8O_7$	504.1690	6.50	503.1617	$[M-H]^-$	85.0275, 87.0067, 111.0066
Flavonoids	Styraxin/Uralenol	$C_{20}H_{18}O_7$	144.0420	3.72	145.0499	$[M-H]^-$	73.0552, 86.0483, 98.1824

The asterisk marks essential amino acids.

3.2. Multivariate discriminant analysis to find metabolite changes along the fermentation process

Individual samples of fresh garlic and fermented garlic collected at different stages during the process (6, 12, 18, 24, 30 and 36 days) were analyzed and a data set was created with the compounds tentatively identified. Firstly, a Partial Least Squares-Discriminant Analysis (PLS-DA) was applied for potential differentiation among samples. This strategy was selected because of the temporal character of the variable under study: the fermentation time. The PLS factors 1 and 2 explained 62.4 and 11.0% of total variability, respectively, that was mostly attributed to the fermentation process, as Fig. 1 shows. Thus, Factor 1 allowed differentiating fresh garlic from fermented garlic sampled at different times. On the other hand, combination of Factor 1 and Factor 2 showed a clear trend of the samples with the fermentation time. The plot reveals that the major changes occurred within the first 6 days of fermentation, but it is also worth noting that the garlic fermented for 36 days reported relevant compositional differences as compared to garlic fermented for 6 days. In contrast, the extracts from garlic fermented for 30 and 36 days were very similar as they were the nearest samples in the plot, suggesting that the most significant changes produced in garlic due to fermentation occurred within the first 30 days of the process. These results are in agreement with those found by Liang et al., who described major changes in garlic occurred within the first 5 days of fermentation, with no significant changes after 25 days of the process [14].

Visualization of the loadings plot associated to the PLS-DA allowed finding two clusters of compounds with high contribution to explain the observed discrimination. The contribution of the three main families of compounds tentatively identified in fresh and processed garlic to explain compositional differences occurring during fermentation is shown in Fig. 2. As can be seen, in both clusters dominated three representative groups of compounds: organosulfur

compounds, amino acids and metabolites, and saccharides and derivatives. Thus, the left cluster that characterizes fresh garlic includes most organosulfur compounds with special emphasis on cysteine derivatives; while disulfides, two sulfoxide derivatives and a sulfothionate were only present in the right cluster. Two additional families of metabolites with particular weight on the PLS-DA test were amino acids and metabolites and saccharides and derivatives. As can be deduced from their position on the PLS bidimensional plot, they were divided into the two clusters. Therefore, it is evident that fermentation drastically affect the three commented families and, for this reason, further studies should focus on them.

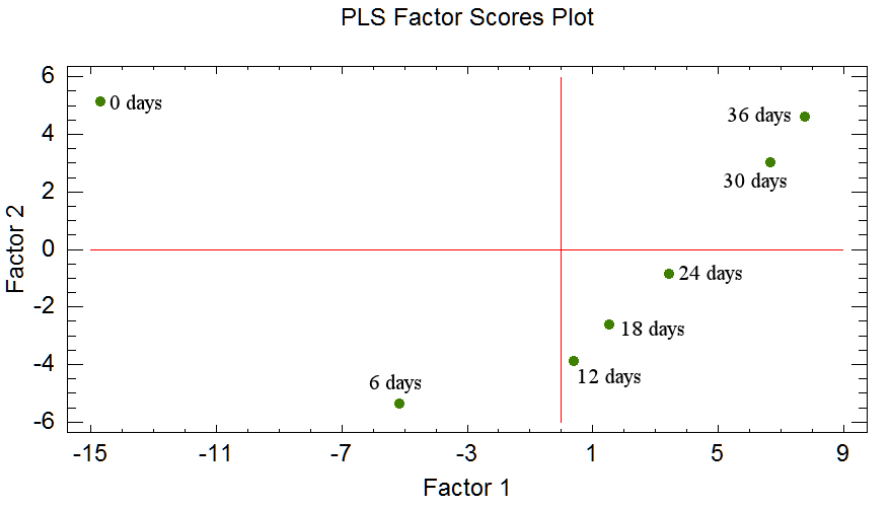

Figure 1. PLS-DA scores plot of the evolution of garlic during the fermentation process.

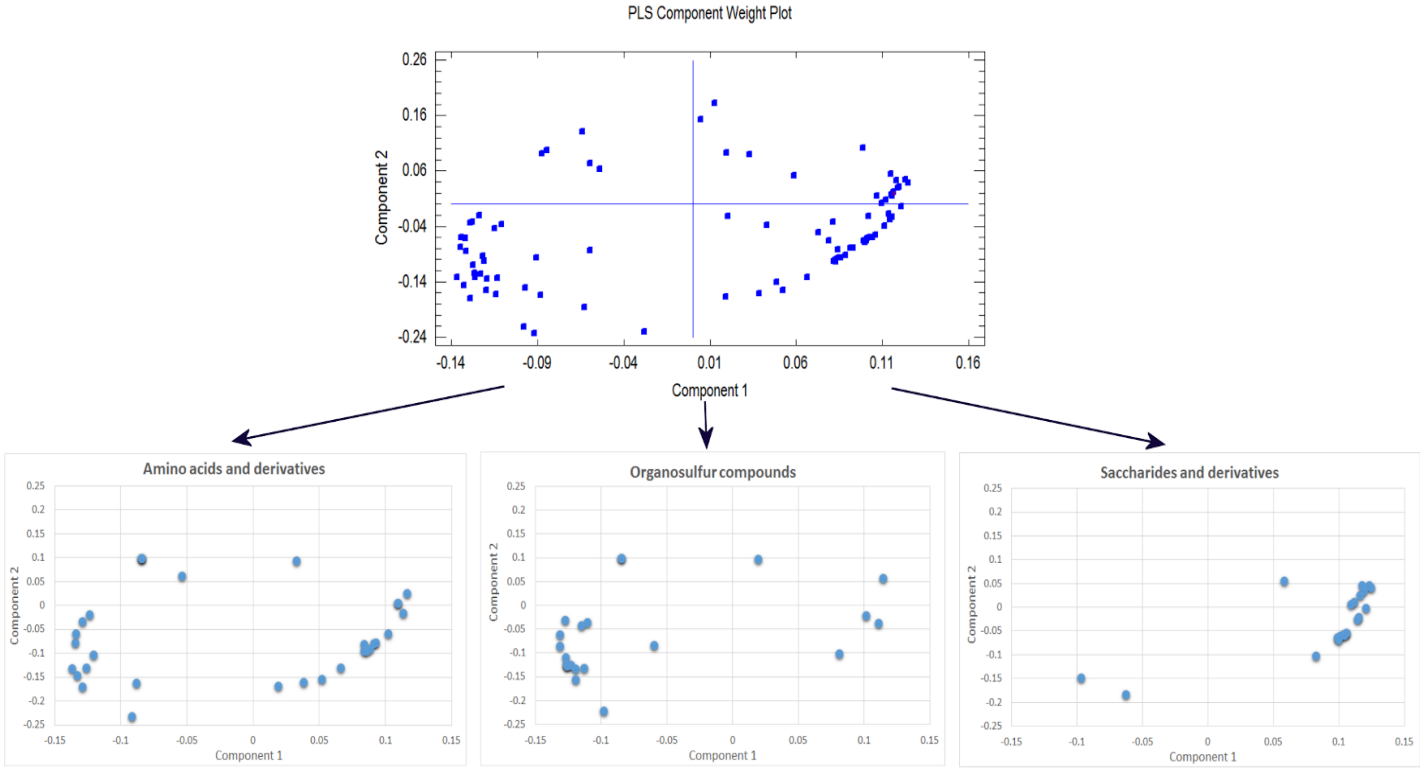

Figure 2. PLS-DA loadings plots of all the identified compounds, and separately the location of the three main families of compounds.

3.3. Changes in organosulfur compounds occurring during garlic fermentation

The previous supervised multivariate analysis was complemented by a Pearson centroid clustering analysis that relates samples collected at different stages as a function of their similarity in the concentration of organosulfur compounds. As can be seen in Fig. 3, garlic samples subjected to fermentation for 30 and 36 days show the highest similarity among the sampling stages, which is in agreement with the results provided by the PLS-DA. Also samples collected at 18 and 24 days are very similar in organosulfur compounds in contrast to the fresh garlic sample and that collected after 6 days of fermentation, which were the most different samples. These results confirm that changes caused by fermentation to organosulfur compounds in garlic occur gradually.

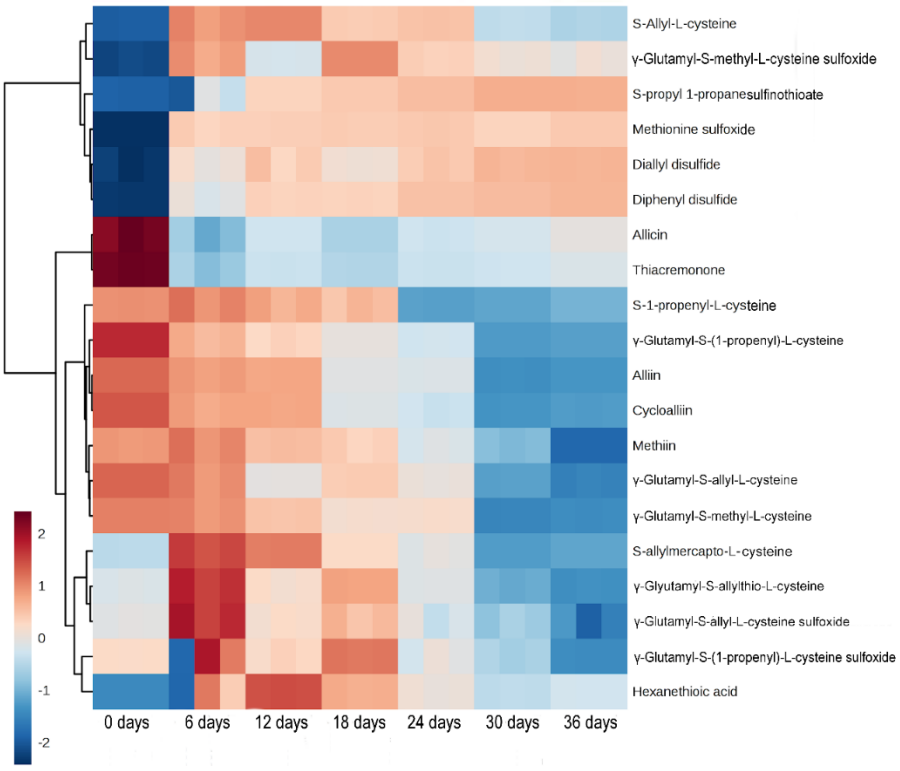

Figure 3. Clustering plot of organosulfur compounds found in fresh (0 days) and fermented garlic at different times (6, 12, 18, 24, 30 and 36 days).

Organosulfur compounds were characterized by two progressive changes: increase or decrease of concentration during the fermentation process. The γ-glutamyl-S-alk(en)yl-L-cysteine family was highly concentrated in fresh garlic, but decreased its concentration along the fermentation process. The same pattern was observed for organosulfur compounds characteristic of fresh garlic such as alliin, cycloalliin or methiin. More drastic was the trend found for allicin and thiacremonone that decreased drastically their concentration after 6 days of fermentation. The high sensitivity of these compounds to heat has been reported in the literature, which shows that allicin is a very unstable compound, rapidly transformed into volatile organosulfur compounds [16]. The opposite situation was observed for disulfide derivatives that increased gradually their concentration during the 36 days of fermentation.

According to Rose et al. [17] and Yoshimoto et al. [18] pathways for synthesis of organosulfur compounds in garlic are proposed in this study as supplementary information (see Supplementary Fig. 2) to interpret metabolic changes occurring during fermentation. The first step is the formation of γ-glutamyl-S-2-carboxypropyl-L-cysteine by condensation of L-cysteine and glutathione with subsequent addition of methylacrylate to the thiol group of the L-cysteine moiety. γ-Glutamyl-S-2-carboxypropyl-L-cysteine is the precursor of the γ-glutamyl-S-(alk(en)yl)-L-cysteine group, being the methyl, 1-propenyl and allyl derivatives the main components of this group. The three compounds were identified in the samples at several fermentation stages. These compounds lead to S-(alk(en)yl)-L-cysteine via deglutamylation or to γ-glutamyl-S-(alk(en)yl)-L-cysteine sulfoxides via S-oxygenation. In the first case, two isomers were tentatively identified in the samples, S-allyl-L-cysteine and S-(1-propenyl)-L-cysteine; while in the second group three main compounds (viz., γ-glutamyl-S-allyl-, γ-glutamyl-S-(1-propenyl)- and γ-glutamyl-S-methyl-L-cysteine sulfoxides) were detected. Both the S-(alk(en)yl)-L-cysteine and γ-glutamyl-S-(alk(en)yl)-L-cysteine sulfoxide

families lead to the formation of sulfoxides by S-oxygenation and deglutamylation, respectively. Members of this family such as S-allyl-L-cysteine sulfoxide (alliin) and S-methyl-L-cysteine sulfoxide (methiin) were tentatively identified in the samples. As reviewed by Lanzotti, sulfoxides are broken under the enzymatic action of allinase and generate intermediate sulfenic acids that condensate to yield thiosulfinates [16]. In this case, only diallyl thiosulfinate (allicin) and S-propyl-1-propanesulfinothioate were detected in garlic samples. Thiosulfinates are very unstable compounds; thus, they are rapidly degraded yielding the large variety of sulfur volatiles present in garlic, where diallyl disulfide and diphenyl disulphide were the tentatively identified compounds within this group.

The normalized area of the organosulfur compounds identified in garlic samples collected at different fermentation stages was plotted to study their evolution during the fermentation process, which allowed determining the effect of fermentation on the corresponding pathways. The results, shown in Fig. 4, revealed that γ-glutamyl-S-(alk(en)yl)-L-cysteine compounds experienced a concentration decrease during fermentation with special emphasis on S-methyl and S-1-propenyl derivatives that were not detected after 30 days of fermentation. The same progressive decrease observed for γ-glutamyl-S-allyl-L-cysteine was found for the three γ-glutamyl-S-alk(en)yl-L-cysteine sulfoxides. On the other hand, differences in behaviour were observed between the two S-alk(en)yl-L-cysteine derivatives in garlic. Thus, while S-allyl-L-cysteine provided a similar concentration in all the garlic samples collected at the different fermentation stages, S-(1-propenyl)-L-cysteine was drastically altered during fermentation as it was not detected after 24 days of fermentation, which fits the results found for its precursor γ-glutamyl-S-(1-propenyl)-L-cysteine.

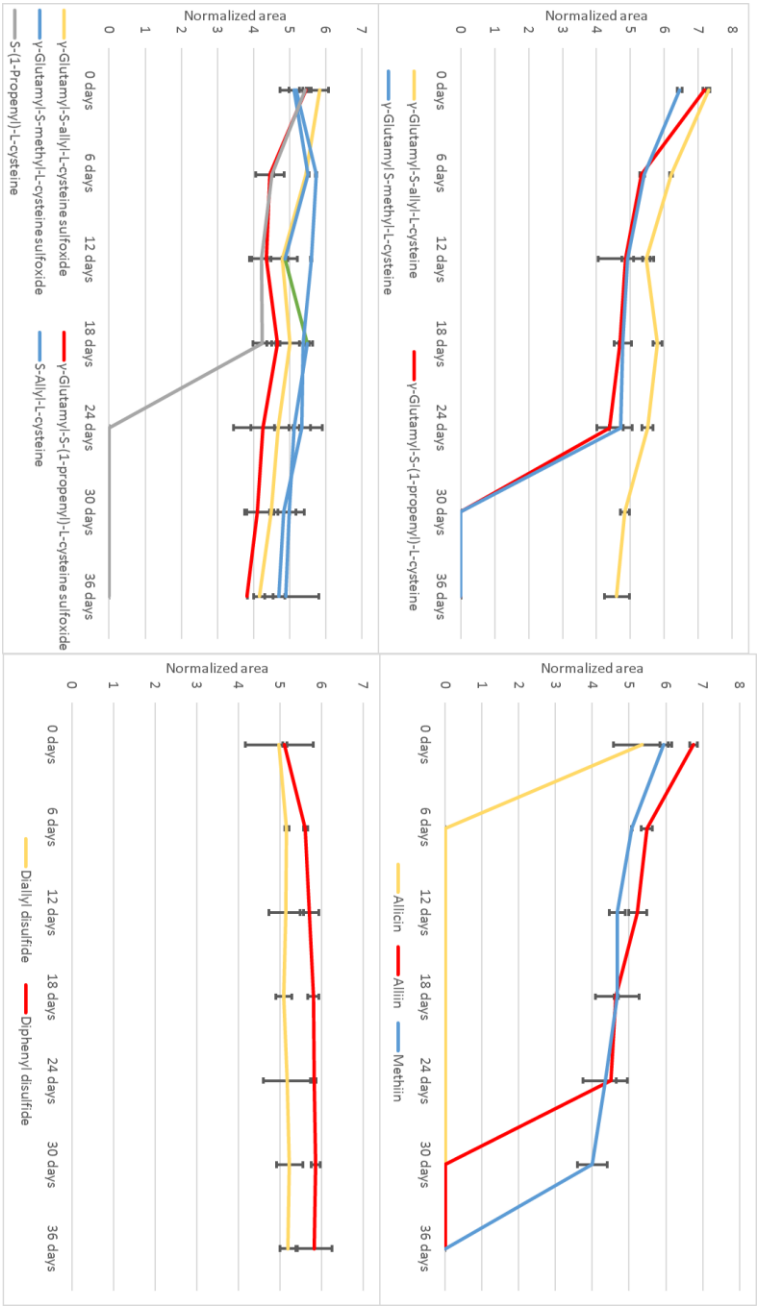

Figure 4. Evolution during fermentation of identified organosulfur compounds involved in the metabolic pathway for synthesis of these compounds. The peak area was normalized by application of logarithm.

Within the sulfoxides group, alliin and methiin were highly concentrated in fresh garlic but they were progressively decreased and completely lost at days 30 and 36, respectively. Allicin, the unique identified compound belonging to the thiosulfinates group, was only detected in fresh garlic. This compound, which is responsible for the aroma of fresh garlic, is formed from alliin by an enzymatic process catalyzed by alliinase when garlic is chopped or crushed. As previously mentioned, allicin is particularly unstable and strongly affected by heat; therefore, the enzymatic pathway is blocked during fermentation. This result agrees with that found by Zhang et al., who stated that allicin disappear in garlic after 10 days of fermentation [19]. In contrast, the volatiles diallyl disulphide and diphenyl disulphide slightly increased their concentration, especially after the first fermentation stage. Therefore, their formation is favored by the fermentation process.

3.4. Changes in amino acids and their metabolites occurring during garlic fermentation

A strategy similar to that followed for organosulfur compounds was adopted for amino acids and metabolites found in fresh and fermented garlic. The Pearson centroid clustering analysis for this group of compounds is illustrated in Fig. 5, which is similar to that obtained for organosulfur compounds since garlic fermented for 30 and 36 days provided a common profile of amino acids and metabolites. Additionally, similar composition of this group of compounds was found in samples fermented for 18 and 24 days, and also in those fermented for 6 and 12 days.

Amino acids and metabolites also reported two different trends for most compounds. Thus, some compounds decreased significantly their concentration during fermentation, among which it is worth mentioning amino acids such as glutamic acid, arginine tryptophan, lysine or asparagine. Other amino acids such as phenylalanine, aspartic acid, proline or leucine/isoleucine increased their concentration although more slightly. Similar behaviour was found for

representative amino acid metabolites during fermentation. Thus, argininic acid, glutamine, tyramine or citrulline decreased considerably their levels in fermented garlic probably owing to metabolism activation under heat and humidity conditions. In contrast, metabolites such as oxoproline, 4-guanidinobutanoic acid or norharman increased their levels during fermentation. This cluster map is also an evidence on the activity of the metabolism of amino acids during garlic fermentation.

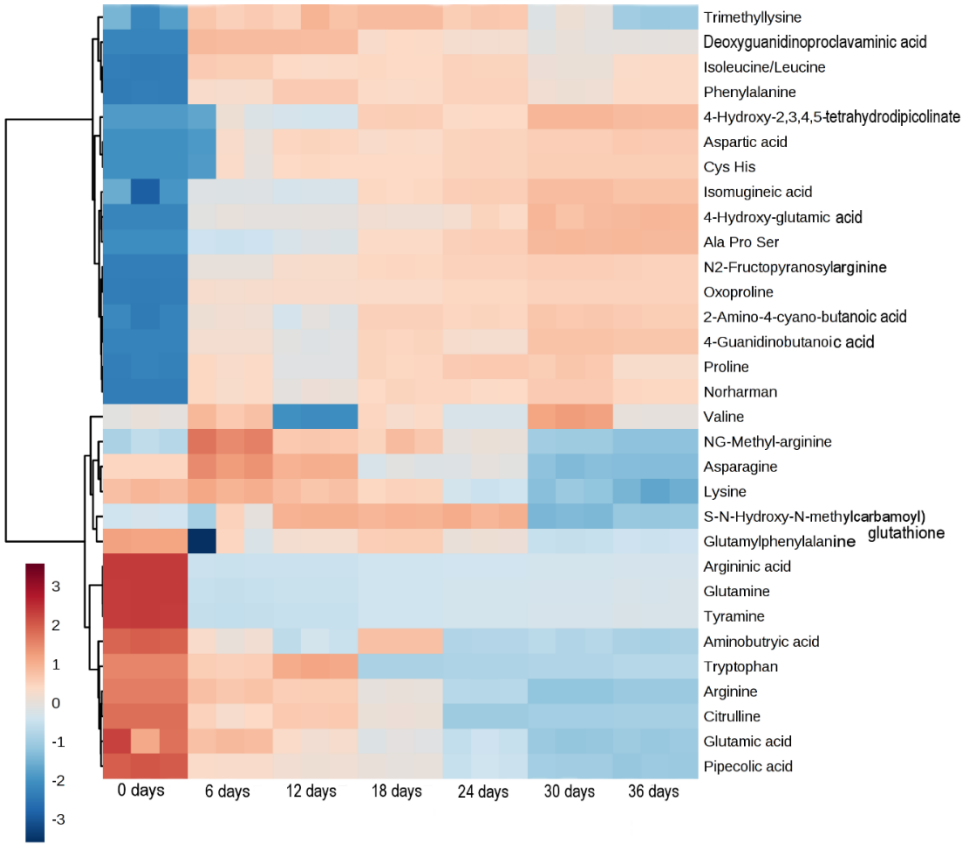

Figure 5. Clustering plot of amino acids and metabolites found in fresh (0 days) and fermented garlic at different times (6, 12, 18, 24, 30 and 36 days).

The behaviour of individual compounds is now discussed as a function of their metabolic connections, for which it is useful to evaluate metabolic pathways

shown in Supplementary Fig. 3. The first interesting result deals with aromatic amino acids such as phenylalanine and tryptophan. Figure 6 illustrates the observed trend for both amino acids as well as for norharman, a β-carboline derivative from tryptophan, and detected in other processed foods such as coffee and cheese. It is worth noting the opposite trends of tryptophan and norharman. Thus, the amino acid is not detected after 18 days of fermentation, while the □-carboline derivative is not detected in fresh garlic, but it is produced once fermentation starts. Simultaneously, phenylalanine increases slightly its concentration during the first fermentation stages, and is then stabilized. Three aromatic amino acids (phenylalanine, tryptophan and tyrosine) are synthesized in the shikimate pathway, which is also responsible for the production of phenolic compounds through phenylalanine. Therefore, garlic fermentation not only removes tryptophan by production of β-carboline derivatives, but also other pathways such as those involving phenylalanine could be activated.

A second pathway significantly altered by garlic fermentation deals with the metabolism of arginine and proline. Arginine decreased slightly its concentration during fermentation. However, citrulline, essentially produced from arginine through the urea cycle, was considerably affected by garlic processing. In fact, citrulline was not detected in garlic during the first 24 days of fermentation, which means that the conversion was blocked. On the other hand, proline, produced after conversion of arginine to ornithine by arginase action, increased slightly its concentration during fermentation, while oxoproline, a proline metabolite, increased significantly its concentration after the first stage of fermentation; then maintained for the rest of the process.

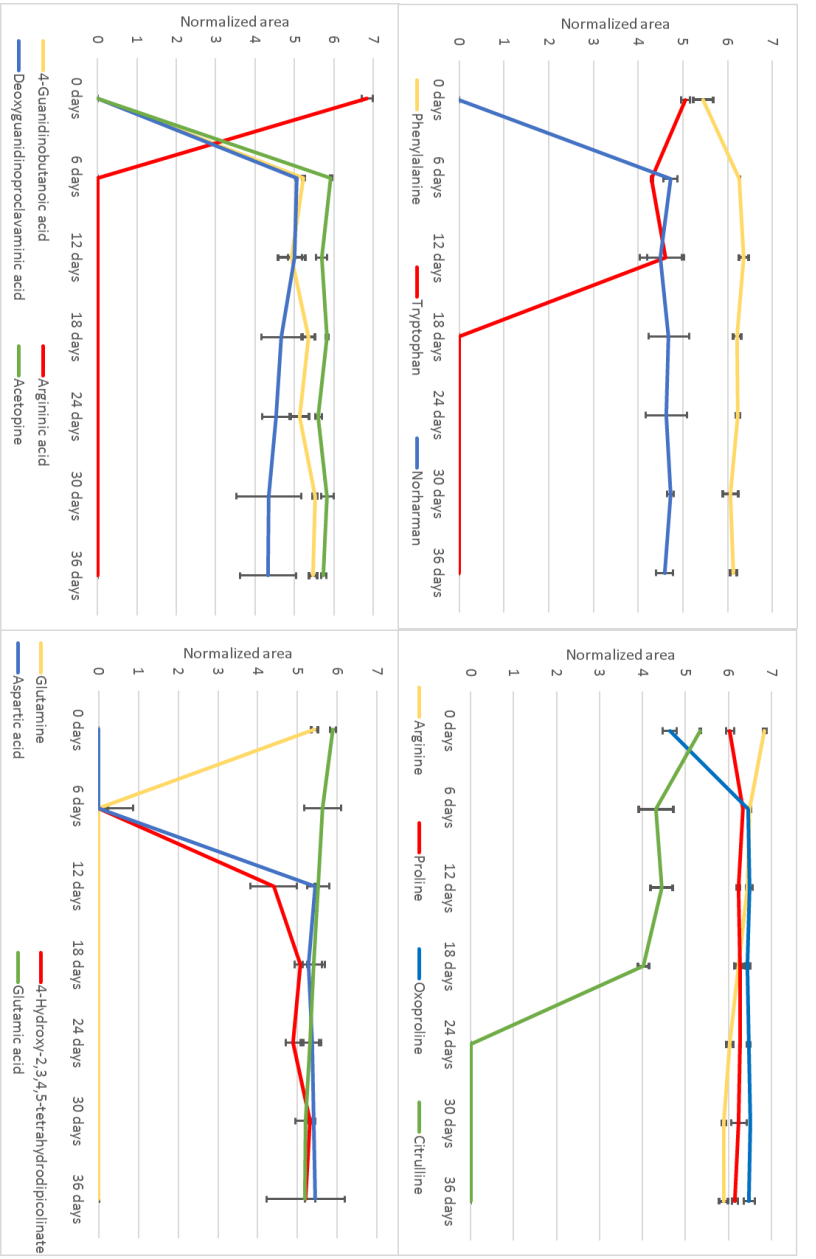

Figure 6. Evolution during fermentation of the main identified amino acids and metabolites affected by the fermentation process. The peak area was normalized by application of logarithm.

Several arginine metabolites were detected in garlic samples with substantial variations in their concentration, indicative of the activation of arginine metabolism during fermentation. Among arginine metabolites, three of them (*viz.*, argininic acid, acetopine and deoxyguanidinoproclavaminic acid) underwent characteristic changes. Thus, argininic acid dissapeared after the first fermentation stage; therefore, it was only detected in fresh garlic. The opposite behaviour was observed for acetopine and deoxyguanidinoproclavaminic acid: both arginine metabolites were not found in fresh garlic and were formed after the first fermentation stage; then, the concentration of both metabolites was stabilized.

Two amino acids involved in pathways directly connected to the citric acid cycle (*viz.*, aspartic and glutamic acids) also changed during fermentation. Aspartic acid and its metabolite 4-hydroxy-2,3,4,5-tetrahydrodipicolinate were formed during fermentation and they were found at day 12 of fermentation; while the concentration of glutamic acid decreased slightly, and its metabolite glutamine, formed by condensation of glutamic acid and ammonia, dissapeared after 6 days of fermentation being only detected in fresh garlic.

3.4. Changes in saccharides and derivatives occurring during garlic fermentation

A common pattern was observed for all saccharides and derivatives identified in garlic samples, which progressively increased their concentration as the fermentation time was longer (in fact, most of them were not detected in fresh garlic). This behaviour suggests degradation of bigger saccharides affected by the Maillard reaction as the origin of them, as demonstrated in our previous research [20]. An identified product of this reaction (5-hydroxymethylfurfural) follows the same pattern, being only detected at day 12 of fermentation and increasing its concentration as fermentation progressed (see Supplementary Fig. 4). These results are in agreement with those from Zhang et al., who demonstrated that 5-hydroxymethylfurfural and reduced saccharides increase their concentration with garlic fermentation, while polisaccharides decrease, confirming development of

the Maillard reaction during garlic fermentation [19,21]. This process is responsible for the caramelization process that change the original color of garlic to that characteristic of black garlic.

4. Conclusions

In this research, polar metabolites in fresh and fermented garlic were tentatively identified using LC−QTOF MS/MS. Ninety-five compounds in polar extracts from the samples were identified and classified in different families, grouped into amino acids and metabolites, organosulfur compounds, saccharides and derivatives, lipids and derivatives, and others. Statistical treatment of the results allowed determining that the main changes were produced during the first 6 days of fermentation, which mainly affected to three families of compounds: organosulfur compounds, amino acids and metabolites, and saccharides and derivatives. Relevant metabolic changes elucidated in the three families mainly contribute to the changes observed in black garlic as a result of the fermentation process.

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The authors declare no conflict of interest.

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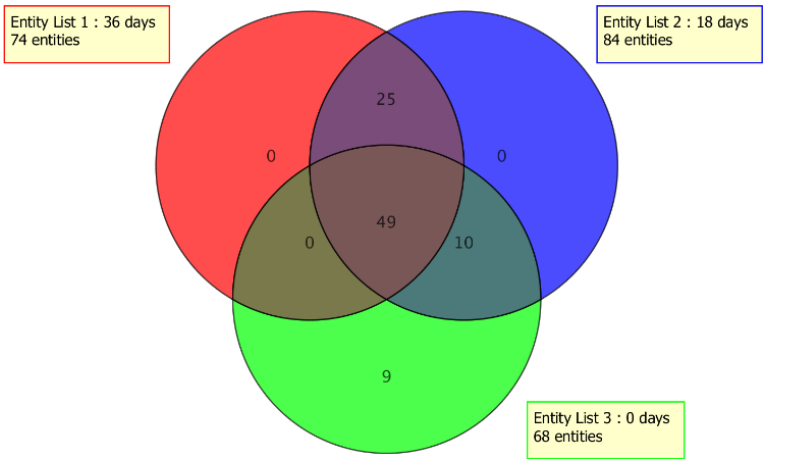
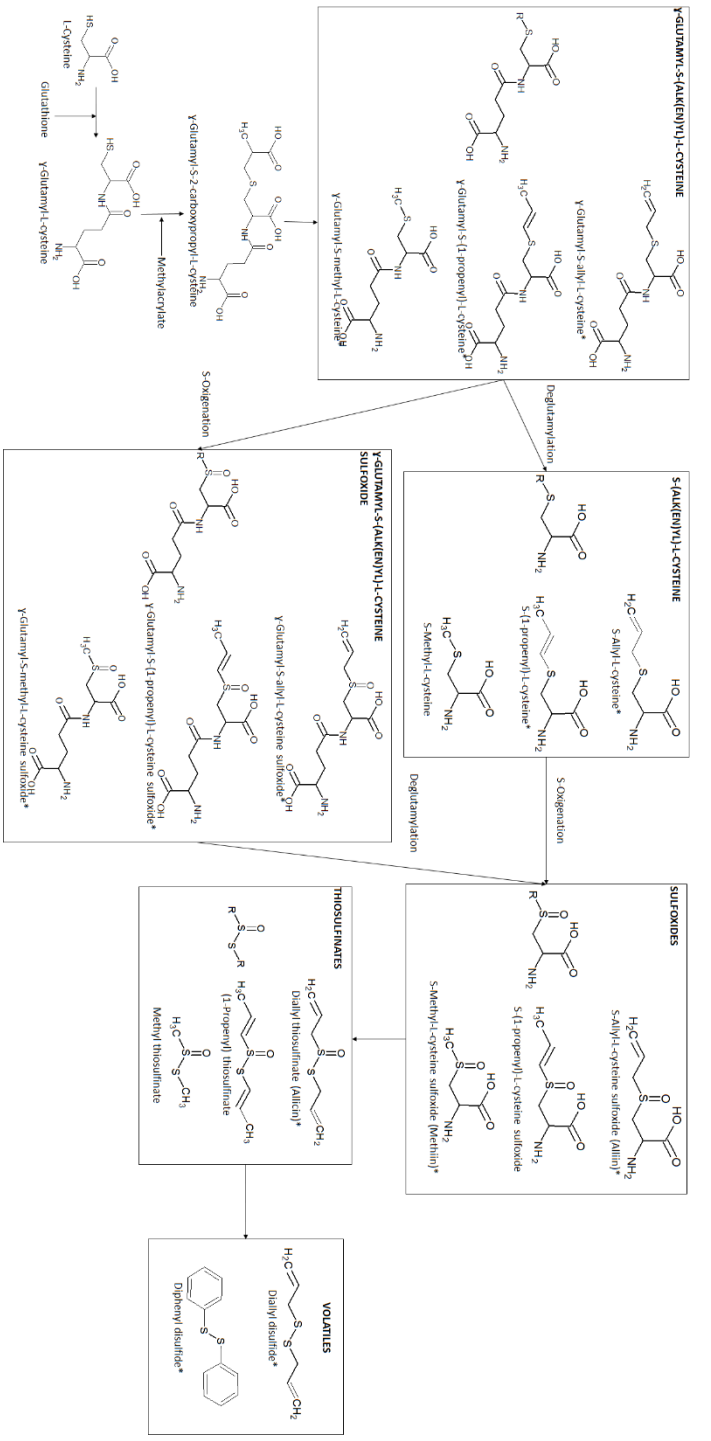
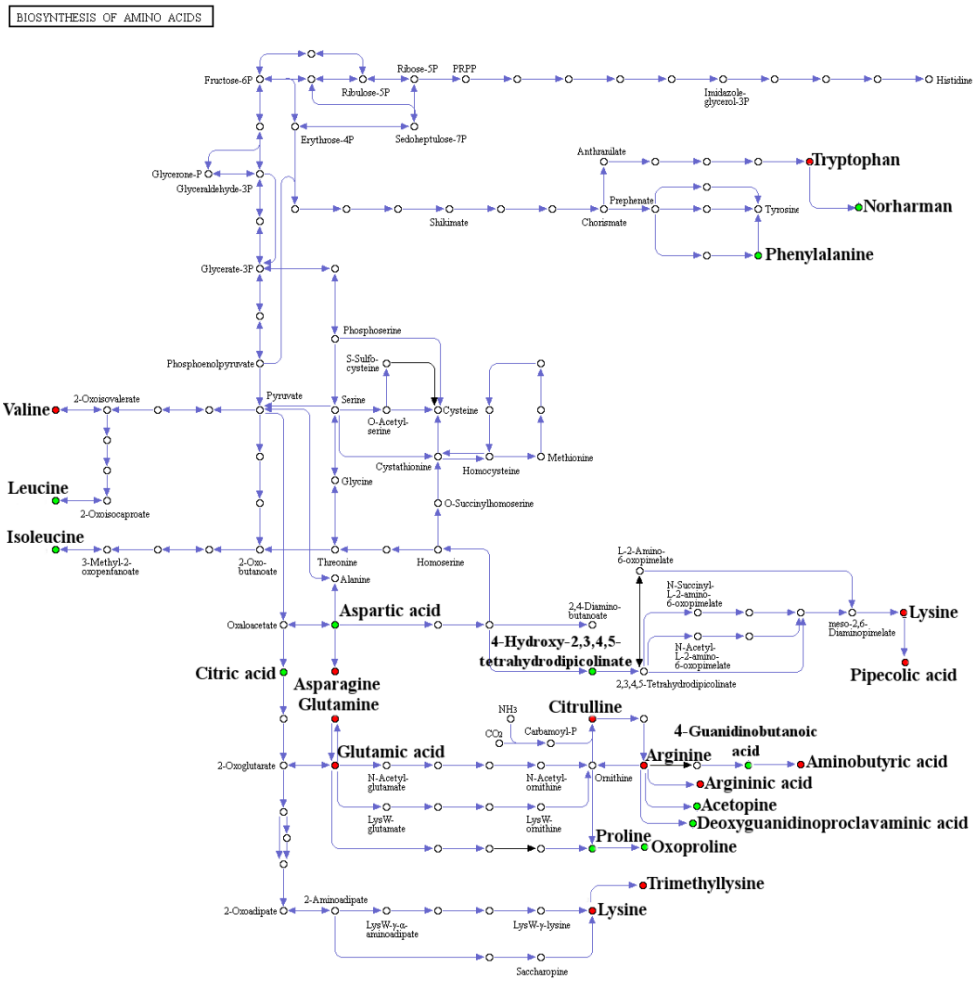
Supplementary information



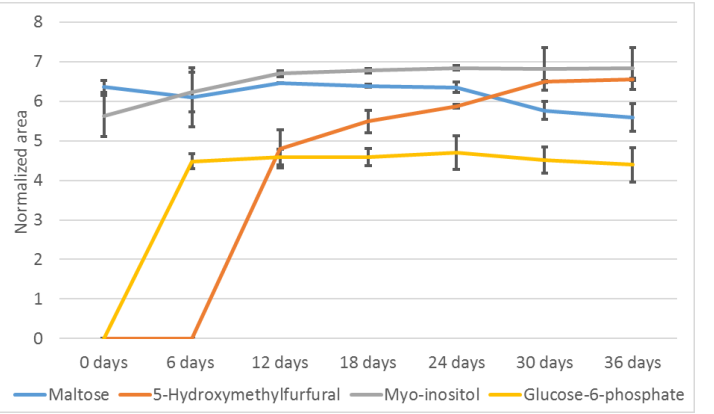
Supplementary Figure 1. Venn diagram of the fresh and black garlic at intermediate (18 days) and final times (36 days) of fermentation.



Supplementary Figure 2. Metabolic pathways of organosulfur compounds synthesis. The compounds identified in these pathways are marked with an asterisk.



Supplementary Figure 3. Metabolic pathways of amino acids synthesis and degradation. The tentatively identified compounds involved in the pathways are marked in green or red depending on the increase or decrease, respectively, of their concentration with fermentation.



Supplementary Figure 4. Evolution with fermentation of some of the identified saccharides and 5-hydroxymethylfurfural. The peak area was normalized by application of logarithm.

DISCUSIÓN DE LOS RESULTADOS

La normativa actual de la Universidad de Córdoba referente a la presentación de la Memoria de la Tesis Doctoral, en la modalidad en la que se incluyen los artículos (publicados o próximos a su publicación) como tales, requiere una discusión conjunta de los resultados de la investigación realizada, cuya estructura depende de su homogeneidad.

La mayor parte de la presente Memoria de Tesis está constituida por la investigación desarrollada, en la que se han empleado técnicas cromatográficas acopladas a espectrometría de masas para el estudio de diferentes matrices vegetales.

La Memoria se ha dividido en tres partes en función de la muestra vegetal estudiada, de modo que la Parte A tiene como objetivo la caracterización de un residuo agroalimentario como es la piel de naranja, la Parte B se centra en la caracterización y cuantificación de compuestos de interés presentes en las hojas de estevia rebaudiana, mientras que en la Parte C se estudian los cambios composicionales durante la fermentación y se caracterizan las fracciones volátil y polar de ajo fresco y negro.

La Parte A recoge un capítulo de libro con el que se adquirieron los conocimientos básicos necesarios para el desarrollo experimental. Además, esta parte discute la puesta a punto de un protocolo de extracción asistido con diferentes tipos de energía para la obtención de compuestos de interés existentes en la piel de naranja y la cuantificación de flavonoides presentes en este desecho

agroalimentario, poniendo así de manifiesto su alto potencial como fuente de compuestos de alto valor añadido.

El objetivo de la investigación que recoge la Parte B fue caracterizar el perfil composicional de las hojas de estevia rebaudiana, identificar las principales familias de compuestos presentes en ellas, cuantificar los principales glicósidos de esteviol en plantas cultivadas en laboratorio, en invernadero y en campo, y estudiar las diferencias en concentración de estos compuestos según se utilice como muestra las hojas o las hojas más las ramas de la planta. Otro objetivo de esta parte de la Memoria fue cuantificar los principales compuestos fenólicos presentes en la hoja y comparar la concentración obtenida en diferentes variedades de la planta.

Por último, la Parte C se centra en el seguimiento de los cambios composicionales que ocurren durante la fermentación del ajo y el efecto en sus rutas metabólicas, así como en la caracterización de los perfiles de compuestos volátiles y polares del ajo fresco y del negro. También se recoge en esta parte el estudio del cambio del perfil de volátiles cuando estos productos se someten a calentamiento.

***Parte A*. Caracterización de residuos agroalimentarios: extracción y análisis de compuestos de alto valor añadido procedentes de la piel de naranja.**

En la Parte de A de esta Memoria se recoge, en primer lugar, una revisión crítica de las diferentes técnicas de extracción y análisis de material agroalimentario, además del desarrollo de estrategias de extracción, caracterización y cuantificación de compuestos de interés presentes en la piel de naranja, el principal desecho de la industria de zumos de este fruto. Esta parte engloba tres capítulos cuyo desarrollo tuvo los siguientes objetivos: (i) conocer y revisar críticamente las diferentes técnicas de extracción de compuestos de alto

valor añadido, así como las herramientas analíticas empleadas en el análisis de material agroalimentario; (ii) proponer un método basado en el uso de energías auxiliares para la extracción de compuestos de interés de la piel de naranja; y (iii) cuantificar los flavonoides presentes en piel de naranja y demostrar el potencial de este desecho para la obtención de compuestos con alto valor añadido.

En primer lugar, se realizó un estudio bibliográfico que proporcionó a la doctoranda la base para los subsiguientes estudios experimentales. En este estudio se revisaron las principales técnicas empleadas en la preparación de la muestra (SLE, LLE, SPE o SPME) para el posterior análisis de compuestos de los compuestos de interés existentes en materiales agroalimentarios. También se discutieron las principales ventajas e inconvenientes del uso de las energías auxiliares —microondas y ultrasonidos— así como de los líquidos sobrecalentados y supercríticos como herramientas para mejorar la extracción de los compuestos de interés.

Se consideraron también las diferentes técnicas de análisis que se han empleado hasta la fecha en esta área, clasificadas en función de su complejidad y de la información que proporcionan. Las técnicas de análisis de una propiedad dada, como la capacidad antioxidante, se usan extensamente en el estudio de material agroalimentario, por lo que se revisaron en profundidad. Por otro lado, las técnicas analíticas más complejas se estudiaron en función del tipo de información que proporcionan, de tal forma que en análisis no orientados se consideraron principalmente la NMR y la MS en análisis directo o acopladas a técnicas de separación previa (CE, LC o GC); mientras que para análisis orientado se consideraron equipos de separación previa acoplados a detectores caracterizados por su sensibilidad, como es el caso del detector de masas de triple cuadrupolo o QqQ.

La redacción de este capítulo proporcionó a la doctoranda una base sólida para soportar la investigación recogida en esta Tesis Doctoral, así como para la

interpretación crítica de la investigación publicada por otros autores.

Con la investigación experimental recogida en esta Parte A de la Memoria la doctoranda pretendió poner de manifiesto el interés de un desecho agroalimentario como es la piel de la naranja para la obtención de compuestos de alto valor añadido.

En primer lugar, se desarrolló un método de extracción usando US como energía auxiliar aprovechando su capacidad para favorecer tanto la formación de emulsiones como la separación de los compuestos de interés de la matriz sólida. Se utilizaron dos extractantes inmiscibles que se emulsionaron por efecto de los US, con lo que se consiguió la extracción simultánea de compuestos polares y no polares. Además, los principales parámetros que afectan a la aplicación de este tipo de energía —amplitud, tiempo de ultrasonicación y ciclo útil— se optimizaron mediante un diseño Box-Behnken de superficie de respuesta para cada uno de los extractos, que culminó con un estudio de deseabilidad en el que se obtuvieron los valores óptimos para el conjunto de los dos extractos, lo que permitió alcanzar la mayor eficiencia en la extracción. La efectividad del método propuesto se demostró comparándolo con uno convencional basado en la maceración, de tal forma que se necesitaron más de 4 horas de maceración para obtener un perfil composicional cuali- y cuantitativo similar al que proporcionó la extracción con US durante 7.5 min.

Los extractos polar y no polar obtenidos se analizaron mediante LC−QTOF MS/MS y se identificaron los compuestos mayoritarios presentes en ellos: principalmente flavonoides y cumarinas, destacando como flavonoides característicos de los cítricos la neohesperidina, hesperetina, naringenina y tangeretina. Estos resultados pusieron de manifiesto la utilidad de los US como energía auxiliar para la extracción de compuestos de alto valor añadido, así como la importancia de la piel de naranja como fuente de estos compuestos.

La subsiguiente previsible etapa en esta a investigación se centró en la cuantificación de los principales flavonoides presentes en la piel de naranja usando un detector de masas de triple cuadrupolo. Para ello, primero se optimizaron todos los parámetros involucrados en la ionización y fragmentación de los compuestos con el fin de obtener una metodología de análisis lo más sensible posible basada en el modo SRM. De esta forma se consiguieron límites de detección y cuantificación en el rango de 0.01−5.0 y 0.01−10.00 ng/mL, respectivamente, para los 16 flavonoides objeto de este estudio; por lo que el método que resultó de esta investigación es uno de los más sensibles que existen actualmente en la bibliografía.

Los flavonoides son compuestos fácilmente extraíbles de matrices vegetales, por lo que se empleó la maceración como técnica de extracción y se estudió la cinética del proceso para determinar el tiempo necesario para obtener el máximo rendimiento. El tiempo óptimo para conseguir este objetivo fue de menos de 30 min ya que tiempos de extracción más largos llevaron a una disminución importante en la concentración de flavonoides en el extracto debida a su degradación por efecto de la acción prolongada de los US.

Una vez puestos a punto el método de extracción y el de análisis, se estudió el estado de la muestra (naranja fresca, liofilizada o secada en estufa) que proporcionaba la mayor concentración de flavonoides en piel de distintas variedades de naranja. La hesperidina, neohesperidina y naringina fueron los compuestos más concentrados en estas variedades, siendo el primero el mayoritario en todos los casos a excepción de la naranja amarga, donde destacaron los otros dos compuestos, lo que se explicó por la acción de dos enzimas diferentes en la ruta de síntesis de los flavonoides. Además, la piel de naranja amarga fue la que presentó un perfil composicional más diferenciado del resto de las variedades.

En cuanto a la influencia del tipo de secado y su comparación con el uso de la naranja fresca, cabe destacar que el secado en estufa produjo una disminución

significativa de la concentración de flavonoides glicosilados y una mayor concentración de las agliconas, especialmente de quercetina; lo que pone de manifiesto que el calor degrada los glicósidos. Por el contrario, la piel liofilizada proporcionó extractos con una composición y concentración similares a los obtenidos con piel fresca, por lo que esta forma de secado preservó en gran medida estos compuestos.

Estos estudios ponen de manifiesto el potencial de la piel de naranja, desecho de la industria de su zumo, como fuente de compuestos de alto valor añadido, así como distintas alternativas para la extracción de dichos compuestos. Además, reflejan la importancia del pretratamiento de la piel, no solo para la cuantificación de estos compuestos en el laboratorio, sino para la obtención de un mayor rendimiento de flavonoides con vistas a su posible uso en diferentes industrias como la alimentaria o la de cosméticos.

Parte B. **Caracterización y cuantificación de metabolitos presentes en las hojas de estevia rebaudiana.**

Esta sección recoge los estudios de las hojas de estevia rebaudiana, un material que ha alcanzado recientemente una gran popularidad por su capacidad edulcorante, pero que había sido poco estudiado fuera del ámbito de la extracción de los glicósidos de esteviol. Por esta razón se emplearon estrategias de análisis no orientadas y orientadas para realizar una amplia investigación sobre esta planta que tuvo los siguientes objetivos: (i) estudiar mediante un análisis no orientado el perfil composicional de las hojas, desconocido hasta la fecha; (ii) cuantificar los principales compuestos presentes en estas hojas y comparar los perfiles de plantas sujetas a diferentes condiciones de cultivo.

El primer objetivo de la investigación recogida en esta parte de la Memoria fue caracterizar los extractos polares y apolares de las hojas de estevia rebaudiana

mediante LC−QTOF en el modo MS/MS. Como resultado, se identificaron tentativamente 89 compuestos con un error de exactitud de masa menor de 4 ppm; la mayoría de ellos en el extracto polar. Solamente los glicerolípidos fueron identificados exclusivamente en el extracto no polar, mientras que los compuestos fenólicos, los terpenoides y los aminoácidos fueron los compuestos mayoritarios en el extracto polar.

Se identificaron 21 compuestos fenólicos, clasificados en dos familias principales: flavonoides y ácidos quínico, cafeico y derivados. La identificación de los flavonoides fue compleja debido a que muchos de ellos son isómeros, pero los mayoritarios (entre ellos quercetina, quercitrina, rutina y quercetina-3-O-arabinósido) pudieron ser claramente identificados. Los ácidos quínico, cafeico y derivados componen una de las familias más importantes identificadas en las hojas de esta variedad de estevia rebaudiana. De los 9 compuestos identificados en este caso cabe destacar 3 ácidos monocafeoilquínicos y 3 dicafeoilquínicos, compuestos a los que se les atribuyen propiedades antimutagénicas, neuroprotectivas, antivirales y antibacterianas. Estos compuestos se caracterizaron por presentar el fragmento 191.0531 *m/z* que corresponde a un residuo de ácido quínico, lo que facilitó la identificación todos los isómeros de ácidos mono- y dicafeoilquínicos presentes en el extracto. La asignación de los picos correspondientes a cada uno de los isómeros se realizó en función de la relación de intensidad con el resto de los fragmentos generados.

Se identificaron también dos familias de terpenoides: diterpenoides y sesquiterpenoides. La mayoría de los diterpenoides identificados fueron glicósidos de esteviol —15 en total—, además de la aglicona del esteviol y la austroinulina (este último un compuesto con propiedades antiinflamatorias previamente detectado en la estevia rebaudiana). Además de los glicósidos de esteviol ampliamente conocidos como el estevióside y los rebaudiósidos, se identificaron dos cuya estructura era desconocida: esteviol+glucosa+4-

metilglucurónido y esteviol+2 glucosas+4-metilglucurónido. El orden de elución de ambos compuestos y su patrón de fragmentación permitió determinar la posición de unión de los azúcares al esteviol y proponer la estructura de ambos compuestos. De los sesquiterpenoides se identificaron 5 isómeros de esterebina, 2 isómeros la esterebina I y J y 3 de los cuatro isómeros E, F, M o N. Sin embargo, estos compuestos no se fragmentaron, por lo que su identificación se basó en la comparación de la masa isotópica obtenida con las bases de datos.

De la familia de los aminoácidos y derivados se identificaron 13 compuestos, de los cuales 5 eran aminoácidos esenciales. La identificación de los aminoácidos se llevó a cabo mediante la búsqueda del ion imonio generado en la fragmentación de estos compuestos. La prolina, colina y serina fueron los compuestos de esta familia más concentrados en las hojas de estevia rebaudiana.

Por último, se identificaron compuestos de otras 6 familias minoritarias: amidas de ácidos grasos y derivados, ácidos grasos y derivados, glicerolípidos, oligosacáridos, purinas y retinoides. Cinco amidas de ácidos grasos se identificaron en ambos extractos de las hojas de la planta en estudio, siendo la docosenamida y la N-estearoyl valina los más concentrados. También en ambos extractos se identificaron 7 ácidos grasos y derivados y 16 glicerolípidos; mientras que se identificaron sólo en el extracto polar 4 oligosacáridos de los que destacan la gamma-ciclodextrina, la purina y un derivado del retinol.

Esta investigación demuestra el gran potencial de las hojas de esta variedad de estevia rebaudiana, no solo para la extracción de edulcorante naturales sino para la obtención de compuestos con un alto potencial beneficioso para el ser humano.

La investigación orientada a la cuantificación de compuestos de estevia rebaudiana se dedicó a las dos principales familias identificadas en los extractos de esta planta: glicósidos de esteviol y compuestos fenólicos, para lo que se

pusieron a punto diferentes métodos basados en LC−QqQ en modo SRM.

Se desarrolló un método analítico para cuantificar 8 glicósidos de esteviol en hojas de plantas cultivadas en laboratorio, en invernadero y en campo, así como para conocer el efecto de incluir las ramas de la planta junto con las hojas como materia prima para la extracción de estos compuestos. En primer lugar se optimizaron todos los parámetros involucrados en la ionización y fragmentación de los compuestos, obteniendo la mayor intensidad en el modo de ionización negativo para todos los glicósidos de esteviol en el que se seleccionaron los aductos $[M–H]^-$ y $[M–Glu]^-$ como precursores. En cuanto a la fragmentación, todos los glicósidos de esteviol produjeron como fragmento mayoritario el generado por la pérdida de un azúcar, ramnosa en el caso del dulcósido A y glucosa en el resto de compuestos.

La optimización de todos estos parámetros dio como resultado un método analítico con una alta sensibilidad, consiguiéndose LODs y LOQs en el rango de 0.1−0.5 and 0.5−1.0 ng/mL, respectivamente: los valores más bajos reportados hasta la fecha en bibliografía para la cuantificación de glicósidos de esteviol.

El método propuesto se utilizó para la cuantificación de estos compuestos en hojas de diferentes variedades de estevia rebaudiana cultivadas en invernadero, en laboratorio y en campo. El esteviósido y rebaudiósido A fueron los compuestos mayoritarios en todas las muestras, alcanzando en algunas de ellas hasta un 55% de la concentración total de glicósidos de esteviol. Las plantas cultivadas en campo contenían una concentración claramente superior al resto, destacando los niveles de esteviósido y rebaudiósido A, B, C y D. Cabe destacar en este grupo de plantas la variedad denominada FIELD3, ya que su concentración en glicósidos de esteviol es considerablemente mayor que en el resto de variedades, a excepción del rebaudiósido C. Comparando estos resultados con los de la bibliografía sobre el poder edulcorante de estos compuestos, se pudo deducir que las variedades más ricas en rebaudiósidos A, B y D proporcionan un mayor poder edulcorante,

destacando la variedad denominada FIELD2 por su relación esteviósido/rebaudiósido A y su alto contenido en rebaudiósido D.

En cuanto a la incorporación de las ramas como materia prima para la extracción de glicósidos de esteviol, hay que poner de manifiesto que globalmente la concentración de estos compuestos fue menor en la muestra que incluía las ramas, especialmente en las variedades denominadas FIELD4 y FIELD7, donde la concentración se redujo más de un 50%. Sin embargo, la variedad FIELD5 no siguió esta pauta ya que su concentración en glicósidos de esteviol fue ligeramente superior en la muestra que incluía las ramas.

Esta investigación permitió desarrollar un método de análisis sensible para el estudio de los glicósidos de esteviol, así como poner de manifiesto que las condiciones de cultivo afectan drásticamente a la concentración de estos compuestos en la estevia rebaudiana, además de demostrar la importancia de una buena selección de la materia prima para mejorar su concentración en los extractos.

El análisis orientado del extracto polar dio lugar a la cuantificación de 29 compuestos fenólicos en las hojas de diferentes variedades de la planta. Al igual que en el caso anterior, se optimizaron todos los parámetros involucrados en la ionización y fragmentación de los compuestos para conseguir un método de análisis sensible. En este caso, dos grupos de compuestos presentaron una tendencia diferente en la optimización de los parámetros de la fuente de ionización: los ácidos cafeoilquínicos requirieron mayores voltajes que los flavonoides para conseguir la máxima altura de pico cromatográfico correspondiente. Esta diferencia justificó su determinación independiente con dos metodologías distintas con el fin de conseguir la máxima sensibilidad para los dos grupos de compuestos. De esta forma se alcanzaron valores de LOD y LOQ en el rango 5−10 y 10−50 ng/mL, respectivamente, para los ácidos cafeoilquínicos; 0.5−25 y 1−50 ng/mL para los flavonoides; y 2.5−25 y 5−50 ng/mL para el resto

de compuestos fenólicos.

Se optimizó el protocolo de extracción de los compuestos fenólicos, que se basó en la extracción por maceración durante 30 min de la muestra con 15 mL de un extractante compuesto por una mezcla metanol–agua 75:25. Para asegurar la completa extracción de los compuestos se realizaron tres re-extracciones del residuo y los extractos generados se analizaron tomando como referencia tres de los compuestos más concentrados. La tercera re-extracción no proporcionó una concentración significativa, por lo que se tuvieron en cuenta solo la primera extracción y las dos re-extracciones siguientes.

Una vez desarrollados los métodos de extracción y análisis, se realizó el estudio de las distintas variedades de estevia rebaudiana. En primer lugar, hay que destacar que los ácidos 1,3-cafeoilquínico, sinápico, 2-, 3- y 4-cumárico, así como el 3-, 4-metilcatecol, la orientina y la homoorientina no se detectaron en ninguna de las muestras y que el ácido cafeico se detectó por debajo de su LOQ en todas ellas. Los ácidos 3,5- y 3,4-dicafeoilquínicos fueron los más concentrados en todas las muestras, a excepción de la variedad *EiretexMorita*, donde los isómeros 3,5- y 4,5- proporcionaron la mayor concentración, alcanzando los 100 mg/g de hoja seca. En el grupo de los flavonoides, la formas glicosiladas de la quercetina (quercetina-3-glucósido, quercitrina, rutina y quercetina-3-arabinósido) se encontraron en una concentración significativamente mayor que el resto de flavonoides, alcanzando, por ejemplo, la quecitrina más de 13 mg/g de hoja seca en la variedad *Eirete*.

El perfil fenólico obtenido para cada muestra permitió estudiar la similitud entre las diferentes variedades en estudio. Las que presentaron una mayor similitud composicional fueron las variedades *Candy* y *Criolla*, mientras que *EiretexMorita* fue la más diferente. Estas diferencias se debieron principalmente a los compuestos mayoritarios (derivados de la quercetina y los ácidos cafeoilquínicos), aunque la apigenina, el kaempferol, la luteolina y sus formas

glicosiladas fueron las que presentaron mayores diferencias entre las variedades, pero su concentración era muy pequeña y, por tanto, sin peso estadístico.

Finalmente se realizó un estudio de correlación entre los compuestos cuantificados. El ácido 4-cafeoilquínico presentó una fuerte correlación con el ácido quínico, lo que parece indicar que este compuesto es el primero de los isómeros formados por la adición de ácido cafeico. También se encontró una relación directa entre los ácidos 5-cafeoilquínico y 4,5-dicafeoilquínico, lo que parece indicar que el primero es el precursor de los ácidos dicafeoilquínicos. Los flavonoides apigenin-7-glucósido y kaempferol presentaron un comportamiento similar ya que la apigenina es el precursor común de ambos compuestos. Por otro lado, la quercetina y la rutina tuvieron un comportamiento opuesto, indicando que la formación de la segunda en las hojas de esta planta se produce consumiendo la quercetina.

Este último estudio de análisis orientado complementa los anteriores y pone de manifiesto el gran potencial de las hojas de estevia rebaudiana para la obtención de compuestos de alto valor añadido como son los compuestos fenólicos, demostrando que están presentes en una concentración que merece tenerse en cuenta.

***Parte C*. Caracterización de la fracción volátil y polar de ajo fresco y negro: evolución durante la fermentación.**

Esta sección recoge los estudios llevados a cabo por la doctoranda para la caracterización del ajo fresco y el ajo negro, ingredientes culinarios tradicional y reciente, respectivamente. Este último está ganando popularidad en la cocina, pero su composición era todavía muy poco conocida al inicio de esta investigación.

Puesto que la composición del ajo fresco se conocía de forma parcial, un primer estudio estuvo orientado a la composición de este condimento, tanto de su

fracción volátil como de la polar, empleando estrategias de análisis no orientado y utilizando diferentes variedades para conocer cómo se modifica el perfil genérico con la variedad.

El siguiente estudio abarcó el perfil del ajo negro en comparación con el ya estudiado del ajo fresco, tanto de la composición de la fracción volátil como de la polar. La enorme diferencia entre los perfiles del ajo fresco y el negro promovió el interés en conocer la evolución que ocurre durante la fermentación de forma exhaustiva y considerando, por supuesto, ambas fracciones.

Como colofón de este estudio y teniendo en cuenta que en su uso culinario tanto el ajo fresco como el negro se someten a calentamiento en muchas ocasiones, se estudió cómo evoluciona la fracción volátil de ambos tipos de ajo en estas condiciones.

Estudio de la composición del ajo fresco

El estudio de la composición del *perfil volátil* del ajo fresco —como los posteriores de la fracción volátil en esta investigación— se realizó mediante GC−MS utilizando un automuestreador de HS.

Se optimizaron los parámetros del HS para obtener la mayor señal cromatográfica en diferentes puntos del cromatograma mediante una superficie de respuesta que combinó los valores obtenidos para varios picos cromatográficos. De esta forma se obtuvieron como óptimos una temperatura de calentamiento de 103 ^{o}C, un tiempo de equilibro del vial de 10 min y un tiempo de inyección de 45 s. Al ser el tiempo óptimo de calentamiento del vial el más largo de los estudiados se realizó una cinética de calentamiento para conocer el efecto de un calentamiento más prolongado. De los compuestos tomados como referencia, todos disminuyeron su concentración con el paso del tiempo, aumentado solamente la acetona y el 2-porpen-1-ol, considerados productos de degradación de otros compuestos. Por ello, se fijaron 10 min como tiempo óptimo de calentamiento.

Una vez optimizado el método de análisis se identificaron 45 compuestos en ajo fresco —de los cuales 17 se identificaron por primera vez en ajo—, que se clasificaron en derivados de la S-alqu(en)il-L-cisteína, compuestos aromáticos y otros. Del primer grupo formaban parte 21 compuestos de los cuales hay que destacar el mono-, di-, trisulfuro de dialilo y el 1,3-ditiano como característicos del ajo fresco. Todos los derivados de la S-alqu(en)il-L-cisteína se caracterizaron por un patrón de fragmentación muy similar, aunque suficientemente diferentes para poder discriminar los isómeros —como los disulfuros de dialilo y de di(1-propenilo)— mediante la intensidad de algunos de los fragmentos generados. En cuanto a los aromáticos, se identificaron un total de 11 compuestos, la mayoría con aromas verde o floral, entre los que cabe destacar 5 derivados del hexano, considerados indicadores de la frescura del ajo.

Se comparó el perfil de compuestos volátiles de tres tipos de ajo (morado, chino y blanco). El di- y el trisulfuro de dialilo fueron los compuestos mayoritarios en todas las variedades, con una concentración relativa entre 42 y 52%. El ajo chino presentó la mayor variedad en volátiles sulfurados, mientras que en el blanco se detectó un mayor número de compuestos aromáticos y el morado presentó la mayor concentración en estos compuestos (15%) debido a la mayor concentración en 2-butenal, el compuesto aromático mayoritario en todas las variedades. Algunos compuestos como el 2-metilbutanal, el 2-pentenal y el 2-etil-2-butenal se detectaron solamente en algunas variedades, por lo que pueden ser considerados compuestos indicadores de esas variedades.

La investigación sobre la *fracción polar* del ajo fresco proporcionó una información de gran interés. La caracterización de esta fracción permitió identificar 82 compuestos, clasificados en aminoácidos y derivados, lípidos y derivados, compuestos organosulfurados, sacáridos y derivados y otros. Hay que destacar la identificación de 11 aminoácidos, de los cuales 5 eran esenciales, así como de algunos compuestos sulfurados característicos de ajo, como la aliina y la

alicina. Los numerosos compuestos organosulfurados identificados junto con la información recogida en la bibliografía permitieron establecer una ruta de síntesis de estos compuestos.

La comparación del perfil de los extractos polares de las variedades antes estudiadas mediante un estudio estadístico permitió detectar que la variedad de ajo chino presentaba un perfil composicional claramente diferenciado de las variedades blanco y morado, más semejantes entre ellas. Las mayores diferencias se encontraron en los aminoácidos y derivados, compuestos organosulfurados y sacáridos y derivados. La valina, asparagina y lisina reportaron una mayor concentración en los ajos blanco y morado, mientras que el comportamiento del triptófano fue el opuesto. En cuanto a los compuestos organosulfurados, todos ellos a excepción de disulfuro de difenilo presentaron una concentración mayor en el ajo morado y blanco. Este último fue el más rico en sacáridos, seguido del chino.

Composición del ajo negro y su comparación con el fresco

El estudio de la *fracción volátil* del ajo negro se realizó mediante el método analítico discutido en el apartado anterior. Se identificaron 51 volátiles, de los que 13 fueron compuestos sulfurados, 13 aromáticos y 3 compuestos sin aroma. De los derivados de la S-alqu(en)il-L-cisteína hay que destacar el sulfuro de metil alilo, con una concentración relativa superior al 18%. Otro de los volátiles mayoritarios en ajo negro fue el furfural, que sobrepasó el 17% y cuya presencia junto con la del 2-acetilfurano y el 5-metilfurfural indican que en la fermentación se produce la degradación de los azúcares mediante la reacción de Maillard. Al comparar los perfiles de volátiles del ajo fresco y el negro se encontraron diferencias significativas. La concentración relativa de la mayoría de los volátiles sulfurados se redujo un 42% en ajo negro, mientras que algunos de estos compuestos no se detectaron. Únicamente el sulfuro de metil alilo incrementó su concentración relativa en el ajo negro. La diferencia en los compuestos aromáticos fue más significativa ya que mientras en ajo fresco la concentración de compuestos que

confieren aroma verde o floral fue significante, la mayoría de volátiles con aroma dulce o tostado (como el furfural y derivados) fueron exclusivos del ajo negro. Estos datos corroboran que los volátiles de aroma verde o floral son indicativos de frescura en ajo, así como que la reacción de Maillard se produce durante la fermentación.

La *fracción polar* del negro mostró una gran riqueza en compuestos polares ya que se encontraron 18 de estos compuestos que no habían sido detectados en ajo fresco. Entre ellos se encontraron la alanina, el norharman y varios derivados de sacáridos; mientras que el triptófano y la alicina, entre otros, no se detectaron en el ajo negro. Al comparar los compuestos polares en ajo fresco y en negro la concentración de 37 de ellos cambió de forma clara entre ambos. El 80% de los aminoácidos y derivados presentaron diferencias significativas, lo que pone de manifiesto que son los compuestos más afectados por la fermentación. De hecho, la concentración de lisina y de arginina fue mucho mayor en ajo fresco, mientras que en ajo negro lo fue la de glutamina, fenilalanina y ácido aspártico. Este comportamiento puede relacionarse con el efecto de la fermentación en las rutas metabólicas en las que están implicados estos compuestos.

Respecto a los compuestos organosulfurados, la mayoría de ellos estaban presentes en el ajo fresco en mayor concentración, mientras que algunos como el disulfuro de difenilo y la tiacremonona se encontraron en mayor concentración en ajo negro. El comportamiento opuesto se observó para los sacáridos y derivados ya que todos —excepto un trisacárido— se encontraron significativamente más concentrados en ajo negro, lo que junto con el gran número de sacáridos exclusivos del ajo negro ponen de manifiesto que los compuestos identificados en este caso proceden de la degradación de sacáridos de mayor tamaño a través de la reacción de Maillard.

Se compararon mediante PCA las variedades de ajo negro procedente de las ya estudiadas en ajo fresco. Se observó que las diferencias entre variedades se

acentúan en el ajo negro ya que la separación en el gráfico es mayor entre estas muestras que en las de ajo fresco. Se encontraron 13 compuestos a una concentración significativamente diferente en los tres tipos de ajo negro, de entre los que destacaron la asparagina, la γ-glutamil-S-metil-L-cisteína, la γ-glutamil-S-(1-propenil)-L-cisteína, la glucosa-1-fosfato y la dihidromacarpina.

Seguimiento de la evolución de la composición del ajo durante el proceso de fermentación

Este estudió se focalizó en la monitorización de los cambios producidos en la composición del ajo durante el proceso de fermentación, desde cero a 36 días. En la *fracción volátil*, los compuestos di- y trisulfuro de dialilo, tomados como referencia del comportamiento de los volátiles sulfurados, sufrieron una disminución drástica en su concentración en los primeros 6 días de fermentación, al igual que los compuestos con aroma verde o floral. Por el contrario, los que se caracterizan por un aroma dulce o tostado incrementaron su concentración exponencialmente a lo largo del proceso, respaldando lo discutido anteriormente sobre la reacción de Maillard. Con los datos obtenidos se creó un PCA para interpretar la evolución de los distintos puntos de la fermentación y los compuestos responsables de la diferenciación entre puntos. Se puso así de manifiesto que los derivados de la S-alqu(en)il-L-cisteína fueron los responsables de la diferenciación del ajo fermentado durante menos de 18 días respecto al fermentado durante 24, 30 y 36 días, mientras que los compuestos aromáticos, especialmente el bencenoacetaldehído, influyeron en la diferenciación del ajo fermentado durante 24 días o más respecto al fermentado durante menos de 24 días.

Los cambios que tienen lugar en la *fracción polar* del ajo durante la fermentación se relacionaron con las alteraciones metabólicas producidas en este proceso. Se identificó un total de 95 compuestos en las muestras tomadas en distintos puntos de la fermentación y a los datos obtenidos se les aplicó un PLS para diferenciar las distintas muestras, obteniéndose una clara progresión desde

el ajo fresco hasta el fermentado durante 36 días. A lo largo del proceso la mayor diferenciación se produjo entre ajo fresco y las muestras obtenidas a los 6 días de fermentación, indicando que los mayores cambios se producen en los 6 primeros días del proceso. Se identificaron los compuestos responsables de la diferenciación entre el ajo fresco y las muestras correspondientes a los primeros días de fermentación (principalmente compuestos organosulfurados), y las muestras correspondientes a los últimos días (prácticamente todos ellos sacáridos y derivados). La influencia de los aminoácidos y derivados en la diferenciación fue similar en los primeros días de la fermentación y en los últimos.

El estudio independiente de los compuestos organosulfurados mostró que su comportamiento se afecta claramente por la fermentación, reduciendo muchos de ellos su concentración a lo largo del proceso hasta estabilizarse a partir de los 24 días de fermentación. Algunos compuestos como la alicina y la tiacremonona disminuyeron drásticamente después de 6 días de fermentación, indicando que se degradan con facilidad en las condiciones de calor y humedad del proceso. Cabe destacar la disminución progresiva de la γ-glutamil-S-(alqu(en)il)-L-cisteína, así como de la aliina y metiina, que no se detectaron en el ajo después de 36 días de fermentación. Por el contrario, los compuestos volátiles disulfuro de dialilo y de difenilo aumentaron ligeramente su concentración a lo largo del proceso, lo que indica que su síntesis está favorecida por las condiciones de fermentación.

En cuanto a los aminoácidos y derivados, el mapa de calor mostró claramente dos agrupaciones de compuestos en función del aumento o disminución de su concentración a lo largo de la fermentación. En estas tendencias cabe destacar el triptófano y su producto de degradación, el norharman, ya que el primero disminuye su concentración hasta no ser detectado después de 18 días, mientras que el segundo no se detectó en ajo fresco, pero sí en ajo fermentado, manteniendo una concentración regular en todos los puntos de muestreo. En cuanto a la arginina y la citrulina, la primera disminuyó su concentración a lo largo del

proceso y la segunda no se detectó a partir de los 24 días de fermentación. Por otro lado, otros metabolitos de la arginina, como el acetopino y los ácidos 4-guanidinobutanoico y deoxiguanidinoproclavamínico, que no se detectaron en el ajo fresco, incrementaron su concentración drásticamente al comienzo de la fermentación. Los resultados obtenidos se relacionaron con las rutas de síntesis/degradación de estos compuestos, determinándose qué reacciones estaban favorecidas o inhibidas por la fermentación.

Por último, el comportamiento de los sacáridos y derivados reveló el mismo patrón: aumentaron su concentración con la fermentación. Se pone así de manifiesto que los compuestos identificados en esta familia se generan durante el proceso mediante la reacción de Maillard por degradación de sacáridos más complejos, confiriendo al ajo negro su olor y color característicos.

Comportamiento del ajo fresco y el negro durante el calentamiento

El último estudio de esta Parte C se centró en evaluar los cambios en la *fracción volátil* cuando el ajo se somete a calentamiento. Se realizó una cinética de calentamiento (8 etapas de calentamiento, desde 10 hasta 120 min, a una temperatura de 103 ºC) de las tres variedades de ajo fresco en estudio con el fin de determinar el efecto del calor en sus perfiles de volátiles. El perfil de volátiles de las tres variedades presentó una tendencia similar en los principales compuestos sulfurados: la concentración del mono- y tetrasulfuro de dialilo aumentó a lo largo del calentamiento, la del di- y trisulfuro de dialilo disminuyó hasta la mitad de la concentración inicial. Algunos compuestos aromáticos como los que confieren aroma dulce o el furfural y sus derivados no se detectaron hasta después de 10 min de calentamiento; lo que se atribuye a la activación de las reacciones de Maillard en el ajo cuando se somete a calentamiento. También cabe destacar que los compuestos con aroma verde o floral disminuyeron drásticamente su concentración, como el 2-butenal, que no se detectó en el perfil volátil de ninguna variedad después de 10−20 min de calentamiento.

Con los datos obtenidos se creó un PCA para evaluar las diferencias de comportamiento de las tres variedades, que mostró que la diferenciación entre variedades se debió principalmente a la Componente 3, donde el sulfuro de dimetilo, la acetona y la 3-penten-2-ona aportaron la mayor contribución a la diferenciación de variedades. Las Componentes 1 y 2, sin embargo, justificaron la separación de los distintos puntos de la cinética de calentamiento, que presentaron la misma tendencia para las tres variedades. Cabe destacar la aportación del 2-butenal a la diferenciación de las muestras sin calentar y la de algunos de los volátiles sulfurados y del 2-pentenal a la diferenciación del ajo calentado durante más de 90 min.

Por último, se comparó el comportamiento cinético del perfil de volátiles del ajo fresco y del negro durante el calentamiento. Se tomaron como modelo del comportamiento de los volátiles sulfurados los mono-, di-, tri y tetrasulfuros de dialilo, que presentaron una tendencia opuesta en ajo fresco y en fermentado durante el calentamiento, siendo el perfil de volátiles del ajo fermentado el que experimentó mayor variación en la concentración de estos compuestos. En el espacio de cabeza del ajo fresco, el di- y el trisulfuro de dialilo disminuyeron progresivamente su concentración relativa y el mono- y el tetrasulfuro de dialilo la aumentaron, mientras que en el perfil de volátiles del ajo negro estos compuestos tuvieron el comportamiento opuesto. Además, la concentración de los volátiles con aroma verde o floral disminuyó durante el calentamiento tanto en el perfil de volátiles del ajo fresco como en el del negro, a excepción del benzaldehído y el bencenoacetaldehído, que se detectaron en el espacio de cabeza del ajo fresco a partir de los 20 min de calentamiento. De los compuestos con aroma dulce o tostado solo alguno de los minoritarios, como el 3-metilbutanal, se detectaron en el perfil de volátiles del ajo fresco calentado. Por otro lado, el furfural y sus derivados aumentaron progresivamente su concentración relativa en el perfil de volátiles del ajo negro durante el calentamiento, probablemente debido a la

acumulación de estos compuestos en el espacio de cabeza.

El PCA creado a partir de los datos obtenidos en la cinética de calentamiento mostró una clara diferenciación en los dos tipos de ajo respecto a los primeros 10 min y el resto del tiempo del proceso. En el caso del ajo negro, los compuestos 3-vinil-1,2-ditiaciclohex-5-eno y benzotiazol contribuyeron más significativamente a la diferenciación de este punto, mientras que el disulfuro de metil alilo, de dimetilo y el furfural fueron los responsables de la diferenciación de las muestras calentadas durante más de 90 min. Por otro lado, el 2-butenal y el 3-metilbutanal determinaron la diferenciación del ajo fresco calentado durante 10 min, mientras que el 2-propen-1-ol, 4-hpetenal y 2-metil-4-pentenal influyeron en la separación en el PCA del ajo fresco calentado durante 120 min.

Los resultados obtenidos en la Parte C de esta Tesis Doctoral suponen la información más completa hasta la fecha sobre la composición de diferentes variedades tanto de ajo fresco como del negro. Además, en ella se han establecido las grandes diferencias composicionales entre el ajo fresco y el negro, así como la presencia de algunos compuestos de interés que aporta la ingesta de ajo negro. También se ha descrito el efecto de la fermentación en la composición de dos familias de compuestos importantes en ajo, lo que abre un camino hacia una fermentación controlada para conseguir un ajo negro más rico en determinados compuestos. Por último, las cinéticas de calentamiento suponen un modelo de comportamiento de las distintas variedades de ajo y de ajo negro cuando se cocinan, estableciendo los distintos matices aromáticos que pueden aportar.

CONCLUSIONES

La investigación recogida en esta Tesis Doctoral se ha centrado en la metabolómica vegetal, poniendo de manifiesto la utilidad de la espectrometría de masas y su capacidad para aportar una gran cantidad de información en el análisis de vegetales. De los resultados obtenidos han derivado las siguientes conclusiones:

- El estudio de la piel de la naranja ha permitido poner de manifiesto que es una importante fuente de compuestos de alto valor añadido, abriendo así la posibilidad de poner en valor estos desechos agroalimentarios y proporcionando las siguientes conclusiones:
  - El uso de ultrasonidos para favorecer la formación de emulsiones de extractantes inmiscibles supone una importante ventaja a la hora de extraer de forma simultánea y rápida los compuestos de interés (polares y apolares) presentes en la piel de la naranja.
  - La identificación tentativa de los compuestos extraídos ha permitido identificar una gran variedad de compuestos en la piel de naranja, entre los que cabe destacar los flavonoides, como la neohesperidina, naringina y tangeretina, y las cumarinas.
  - El estudio de la piel de diferentes variedades de naranja permitió establecer diferencias significativas en su concentración de flavonoides, correspondiendo el perfil más diferenciador a la naranja amarga.
  - La comparación de dos técnicas de secado, liofilización y secado en estufa, puso de manifiesto que este pretratamiento afecta a los compuestos de la piel de naranja, especialmente el secado en estufa ya que el calor favorece

la degradación de los flavonoides glicosilados, por lo que aumenta la concentración de las formas aglicona.

- El estudio de la estevia rebaudiana recogido en esta Tesis Doctoral ha permitido establecer el perfil composicional de las hojas de esta planta, así como cuantificar compuestos pertenecientes a las principales familias en diferentes variedades de estevia rebaudiana, concluyendo que:
  - La identificación de un total de 89 compuestos polares y apolares en las hojas de estevia rebaudiana ha puesto de manifiesto la variedad en la composición de esta planta, no solo en edulcorantes sino también en otros compuestos de interés.
  - Las familias más importantes identificadas en la estevia rebaudiana fueron los glicósidos de esteviol, ampliamente conocidos por su poder edulcorante, y los ácidos quínico, cafeico y derivados, donde cabe destacar los ácidos cafeoilquínicos, compuestos con una gran capacidad antioxidante.
  - El estudio de la concentración de glicósidos de esteviol en las hojas de esta planta mostró que el esteviósido y el rebaudiósido A son los compuestos mayoritarios, independientemente de la variedad o del tipo de cultivo.
  - El tipo de cultivo de la estevia rebaudiana influye drásticamente en la concentración de glicósidos de esteviol en las hojas, obteniendo una mayor concentración en la cultivada en el campo.
  - Las ramas de la planta tienen, en general, una menor concentración de glicósidos de esteviol que las hojas.
  - Los compuestos fenólicos, especialmente los ácidos cafeoilquínicos y los flavonoides derivados de la quercetina, están presentes en una alta concentración (del orden de los mg/g de hoja seca) en las hojas de estevia rebaudiana.

- La variedad *EiretexMorita* es la que contiene una mayor concentración en ácidos cafeoilquínicos y un perfil fenólico más diferente al resto de variedades, mientras la variedad *Eirete* tiene una mayor concentración en flavonoides.

- La investigación recogida en esta Memoria sobre el ajo fresco y negro ha permitido realizar una caracterización de la fracción volátil y polar en ambos productos, así como estudiar los cambios composicionales que se producen tanto en la fermentación como en un calentamiento prolongado. De esta forma, las conclusiones que se obtuvieron de este estudio son:
  - La identificación en ajo fresco de 45 compuestos volátiles —entre los que destacan los compuestos organosulfurados y aromáticos— y de 82 compuestos polares, muchos de ellos aminoácidos y derivados, pero también compuestos organosulfurados.
  - La concentración de los diferentes compuestos identificados, especialmente los aminoácidos y compuestos oragnosulfurados tanto volátiles como polares, permite diferenciar las tres variedades diferentes de ajo estudiadas.
  - Los 51 compuestos volátiles y 93 polares identificados en ajo negro, muestran que algunos son exclusivos del ajo negro (como los que le confieren los aromas dulce y tostado), lo que pone de manifiesto los cambios producidos durante la fermentación.
  - La fermentación disminuye progresivamente la concentración de compuestos organosulfurados tanto volátiles como polares.
  - La concentración de aminoácidos y derivados cambia de forma notable durante la fermentación.
  - El comportamiento del furfural y derivados y de los azúcares y derivados muestra que la fermentación produce la activación de la reacción de

Maillard a lo largo del proceso.

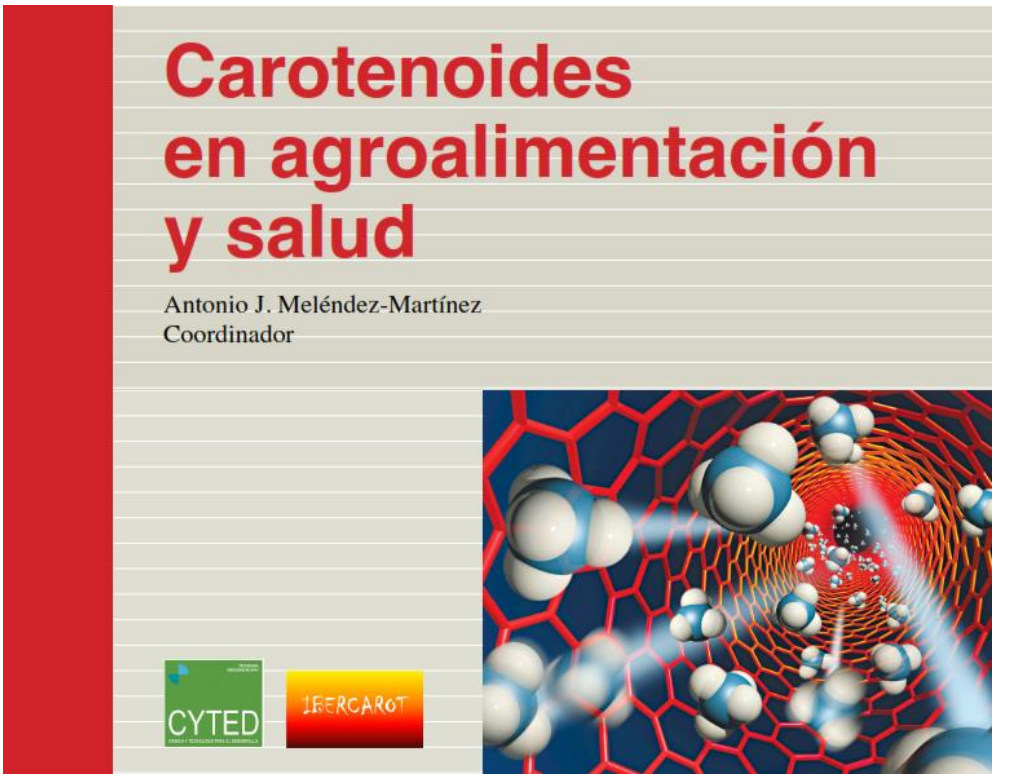
- El estudio de las tres variedades de ajo fresco y de ajo negro cuando se someten a calentamiento permite extrapolar los cambios que se producen en la fracción volátil a los que experimentarán durante el cocinado.

ANEXOS

ANEXO I

Capítulos de libro de los que la doctoranda es co-autora

- Estrategias en metabolómica para el estudio de los carotenoides en plantas y su acción en el organismo humano.
  María Dolores Luque de Castro, **María Molina-Calle**
  Editorial Terracota, México, 2016.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Estrategias en metabolómica para el estudio de los carotenoides en plantas y su acción en el organismo humano

María Dolores Luque de Castro y María Molina-Calle

La metabolómica como marco de estudio de los carotenoides

El auge de la metabolómica en la última década ha hecho que esta disciplina adquiera un papel cada vez más importante en estudios sobre la elucidación de mecanismos, rutas y comportamiento de sistemas vivos, así como en estudios sobre la interacción de estos sistemas con el medio. En sus comienzos, esta nueva "ómica" fue definida por Fiehn (2002) como "un análisis global, exhaustivo, en el que se identifican y cuantifican todos los metabolitos de un sistema biológico". Desde entonces, la metabolómica se ha definido de diferentes formas en numerosos artículos y revisiones científicas, por lo que Beyoğlu e Idle (2013) aunaron todas ellas en una definición amplia de la nueva disciplina como "el estudio global y no sesgado del conjunto de moléculas pequeñas (<1 kDa) en un biofluido, tejido, órgano u organismo".

La metabolómica está directamente relacionada con el resto de las ómicas como

ANEXO II

Comunicaciones a congresos y reuniones

- Metabolómica vegetal aplicada a productos de alto valor añadido y aprovechamiento de residuos agroalimentarios.

  María Molina-Calle, Feliciano Priego-Capote, María Dolores Luque de Castro.

  IV Congreso Científico de Investigadores en Formación de la Universidad de Córdoba. Córdoba (España), 2014.

  Tipo de evento: Comunicación oral **Ámbito**: Nacional

- Estevia: Cuantificación de glicósidos de esteviol mediante LC−QqQ e identificación de otros compuestos mediante LC−QTOF.

  María Molina-Calle, Feliciano Priego-Capote, María Dolores Luque de Castro.

  XV Reunión del Grupo Regional Andaluz de la Sociedad Española de Química Analítica. Almería (España), 2016.

  Tipo de evento: Comunicación oral **Ámbito**: Nacional

- Caracterización de ajo fresco y fermentado mediante LC−QTOF MS/MS: cambios composicionales durante la fermentación.

  María Molina-Calle, Feliciano Priego-Capote, María Dolores Luque de Castro.

  IV Congreso Científico de Investigadores en Formación de la Universidad de Córdoba. Córdoba (España), 2016.

  Tipo de evento: Comunicación oral **Ámbito**: Nacional

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SEDE CÓRDOBA

Córdoba, 18 y 19 de Noviembre de 2014
En el Rectorado de la Universidad de Córdoba
Avda. Medina Azahara 5

METABOLÓMICA VEGETAL APLICADA A PRODUCTOS DE ALTO VALOR AÑADIDO Y APROVECHAMIENTO DE RESIDUOS AGROALIMENTARIOS

María Molina-Calle, Feliciano Priego-Capote, María D. Luque de Castro

OBJETIVOS

La necesidad de un mejor aprovechamiento de nuestros recursos agrícolas en general, y de los desechos de la industria agroalimentaria en particular, es un tema candente de los países emergentes, pero también de los desarrollados, como muestra la nueva convocatoria de proyectos de la Unión Europea "Horizonte 2020" [1]. Uno de los tópicos de esta convocatoria, el "Waste7", está orientado "a asegurar un uso sostenible de los desechos de la agricultura, de sus co-productos y sub-productos". En este ámbito se incluyen sectores característicos de la dieta mediterránea, como la vid/vino y el olivo/aceite, o incluso los cítricos, pero también otros sectores tradicionales (como el del ajo en su versión convencional o alterada —ajo negro, por ejemplo), o sectores emergentes que, provenientes de otros continentes, están asentándose entre nuestros cultivos, como el de estevia. Por otra parte, la metabolómica es una herramienta clave en el desarrollo de la agricultura, en la mejora de sus productos y en el aprovechamiento de los residuos derivados, por lo que (a través del estudio de los metabolitos, tanto primarios como secundarios) puede aportar información crucial por ella misma o asociada a la obtenida mediante otras ómicas, como la genómica, transcriptómica y/o proteómica (biología de sistemas) [2].
Teniendo en cuenta el interés actual en el aprovechamiento de estas materias primas y la utilidad de la metabolómica en este ámbito, los objetivos de la investigación que se presenta fueron:

- Valorizar los residuos generados en la producción de zumos de naranja: identificar compuestos que presenten un alto valor añadido para el sector agroalimentario u otros sectores (cosmético, farmacéutico, etc.); proponer métodos de extracción eficaces y de bajo coste; cuantificar dichos compuestos y estudiar la influencia del

método de secado de los residuos como etapa previa a la extracción y comparar entre variedades de naranjas y clementinas [3].

- Estudiar el perfil volátil de distintas variedades de ajo blanco y ajo negro; caracterizar de forma exhaustiva los extractos obtenidos con el fin de identificar las rutas metabólicas afectadas en la obtención del ajo negro y determinar compuestos con una posible acción biológica (saludables para el organismo).
- Optimizar el proceso de extracción de edulcorantes naturales existentes en la planta estevia (esteviósidos) y proponer un método eficaz de purificación. Determinar las rutas metabólicas implicadas en la generación de los esteviósidos, cuantificar dichos compuestos en distintas variedades de estevia e identificar otros compuestos de interés presentes en ella para un aprovechamiento integral de la planta [4].

MÉTODO DE TRABAJO

Para alcanzar los objetivos descritos anteriormente se han empleado diversas metodologías de trabajo basadas en la extracción, separación y análisis de los compuestos de interés en cada caso, utilizando para ello técnicas de vanguardia en metabolómica.

Para el estudio de los desechos de naranja (mayoritariamente consistentes en la piel) se empleó una emulsión–extracción asistida por ultrasonidos (USAEE) en la que, con la ayuda de los ultrasonidos, se crea una emulsión de dos extractantes no miscibles (n-hexano y una mezcla metanol–agua 80/20 v/v) que favorece la transferencia de compuestos de diferente naturaleza al extractante con mayor afinidad. Se optimizaron los parámetros de sonicación: amplitud de sonicación del 20%, ciclo útil de 0.75 s/s y 7.5 min de tiempo de extracción. La separación y el análisis de los compuestos presentes en estos extractos (separados previamente mediante centrifugación) se llevaron a cabo mediante un cromatógrafo de líquidos acoplado a un espectrómetro de masas de tiempo de vuelo (LC–QqTOF). Una vez identificados, los compuestos de mayor interés (flavonoides) se cuantificaron utilizando como materia prima la piel de distintas variedades de naranja y clementinas usando cromatografía líquida acoplada a un espectrómetría de masas de triple cuadrupolo (LC–QqQ).

La separación y el análisis del perfil de volátiles en ajo blanco y ajo fermentado (o ajo negro) se llevaron a cabo mediante un cromatógrafo de gases acoplado a un espectrómetro de masas (GC–MS). Se utilizó un muestreador de espacio de cabeza con para analizar exclusivamente la atmósfera generada al calentar el ajo. Se optimizaron los parámetros de calentamiento: temperatura del vial de 103 ºC, tiempo de equilibrio del vial de 10 min y tiempo de inyección de 0.8 min. Por otro lado, se realizó una extracción de ajo blanco y negro mediante maceración usando como extractante una mezcla metanol–agua al 50%. Los extractos obtenidos se analizaron mediante LC–QqTOF con el fin deg caracterizar los metabolitos presentes en cada tipo de muestra.

Para la optimización de la extracción de esteviósidos a partir de la hoja de la planta, se ensayaron distintas técnicas de extracción usando etanol–agua como extractante: maceración, reflujo, extracción con líquidos sobrecalentados (SHLE), extracción asistida por microondas (MAE) y extracción asistida por ultrasonidos (USAE), dando lugar a rendimientos similares en todos los casos. Por ello se seleccionó la maceración debido a su simplicidad, rapidez y bajo coste y se optimizaron los principales parámetros de extracción: proporción etanol–agua, tiempo, relación muestra–extractante y pH. Una vez establecido el método de extracción, se aplicó a distintas variedades, obteniendo extractos que se analizaron por LC–QqQ para la cuantificación de esteviósidos y por LC–QqTOF para la identificación de otros compuestos presentes en los extractos que pudieran ser de interés.

APORTACIONES

Las aportaciones de esta investigación abarcan tanto el establecimiento de protocolos de extracción optimizados para los compuestos de interés como la determinación y cuantificación de dichos compuestos.

Así, se han establecido métodos de extracción efectivos para la extracción de compuestos de alto valor añadido a partir de desechos de la producción primaria, como es la piel de naranja, y a partir de potenciales cultivos, como es la estevia. La USAEE ha proporcionado un método de extracción rápido, eficaz y exhaustivo de toda la gama de metabolitos presentes en la piel de naranja (compuestos polares y no polares), siendo una alternativa a tener en cuenta para la extracción conjunta de aceites esenciales y antioxidantes. Para la

extracción de esteviósidos a partir de la estevia se ha establecido un protocolo de extracción donde todas las variables a tener en cuenta han sido optimizadas con el fin de lograr una mayor efectividad en el proceso de extracción. La simplicidad y rapidez de este protocolo permitiría su extrapolación a escala industrial mejorando así la eficiencia de extracción de esteviósidos.

En esta investigación se han determinado metabolitos con importantes características, como son los flavonoides procedentes de la piel de naranja (tales como hesperidina, neohesperidina, tangeretina o naringenina), que han demostrado poseer una gran capacidad antioxidante; y una gran variedad de esteviósidos (esteviósido, reabudiósidos o dulcósido), que poseen un poder edulcorante entre 100 y 500 veces superior a la sacarina. Dichas características hacen que estos compuestos tengan un alto valor añadido y, por consiguiente, la explotación de estas materias sea aprovechable no sólo para la industria alimentaria, sino también para la farmaceútica o la cosmética.

La cuantificación de estos compuestos ha permitido estudiar la diferenciación entre variedades y la influencia de procesos de secado de la materia prima. En el caso de la piel de naranja, se ha demostrado que el secado en estufa provoca una disminución del contenido en flavonoides con respecto a la piel fresca debido a la degradación de estos compuestos, siendo la liofilización una técnica menos agresiva ya que el contenido en flavonoides se ve menos afectado. El estudio de flavonoides en piel de distintas variedades de naranja y clementinas puso de manifiesto una clara diferenciación entre ellas debida al contenido total en flavonoides y al contenido relativo de cada uno de ellos. La cuantificación de esteviósidos, por otro lado, permitió determinar qué variedades presentan un mayor contenido en dichos compuestos y dilucidar cuáles tienen un mayor poder edulcorante en relación a su contenido en esteviósidos.

Uno de los siguientes estudios a realizar para continuar esta investigación será la identificación de otros compuestos presentes en las hojas de estevia que se extraen junto con los esteviósidos, que podrían presentar otras propiedades de interés (lo que es muy probable dadas las cualidades saludables y curativas que se le atribuyen). Por otro lado, aunque algunos compuestos derivados de la aliina ya han sido identificados en la fracción volátil de ajo blanco y negro, es necesario profundizar en esta identificación, así como en la de compuestos de interés en los extractos obtenidos a partir de estos ajos. Esto permitirá ver

la evolución del metabolismo que experimenta el ajo a lo largo del proceso de fermentación para obtener el ajo negro, así como el comportamiento de distintas variedades de ajo bajo este proceso.

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ACREDITA que :

MARÍA MOLINA CALLE

ha presentado **la COMUNICACIÓN ORAL** que lleva por título :

METABOLÓMICA VEGETAL APLICADA A PRODUCTOS DE ALTO VALOR AÑADIDO Y APROVECHAMIENTO DE RESIDUOS AGROALIMENTARIOS

en el **III Congreso Científico de Investigadores en Formación en Agroalimentación ceiA3,** organizado por la Escuela Internacional de Doctorado en Agroalimentación eidA3, celebrado en Córdoba los días 18 y 19 de noviembre de 2014.

Y para que así conste, se expide y firma este certificado en Córdoba, a 19 de noviembre de 2014

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Rectorado de la Universidad de Córdoba – Avd. Medina Azahara 5, 14071 CÓRDOBA

XV REUNIÓN DEL GRUPO REGIONAL ANDALUZ DE LA SOCIEDAD ESPAÑOLA DE QUÍMICA ANALÍTICA

Almería, 30 de junio y 1 de julio de 2016

GRUPO REGIONAL ANDALUZ
SOCIEDAD ESPAÑOLA DE
QUÍMICA ANALÍTICA

UNIVERSIDAD DE ALMERÍA

CO-05

ESTEVIA: CUANTIFICACIÓN DE GLICÓSIDOS DE ESTEVIOL MEDIANTE LC–QqQ E IDENTIFICACIÓN DE OTROS COMPUESTOS MEDIANTE LC–QTOF

María Molina-Calle[1,2,3], Feliciano Priego-Capote[1,2,3], María Dolores Luque de Castro[1,2,3]

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La estevia ha ganado recientemente popularidad gracias a su sabor dulce, lo que la ha convertido en una buena alternativa a los edulcorantes artificiales. Los compuestos responsables de esta propiedad son los denominados glicósidos de esteviol, formados por esteviol (un diterpeno) enlazado a distintos azúcares. Estos compuestos han sido aprobados recientemente como aditivos alimentarios seguros, por lo que existe un creciente interés en su estudio. Sin embargo, la gran mayoría de estudios publicados centra su atención en los glicósidos de esteviol y poco se ha incidido en otros compuestos presentes en estevia que pueden aportarle un valor añadido.

La investigación aquí presentada consta de dos partes: la cuantificación de glicósidos de esteviol en estevia cultivada en el sur de España y la identificación de otros compuestos presentes en la hoja de la planta. Primero, se optimizó el procedimiento de análisis de los glicósidos de esteviol, en el que se usó un cromatógrafo de líquidos acoplado a un espectrómetro de masas de triple cuadrupolo (LC–QqQ). Con el método propuesto, se alcanzaron límites de cuantificación y de detección de 0.5 y 0.1 ng/mL respectivamente —los menores reportados en bibliografía; por tanto, es el método más sensible propuesto hasta ahora para el análisis de estos compuestos. Se analizaron muestras de hojas de estevia cultivada en laboratorio, en invernadero y en campo, obteniéndose una concentración significativamente mayor en las cultivadas en campo. Por último, se evaluaron las hojas frente a las hojas más los tallos de la planta como material para la extracción de los glicósidos de esteviol, obteniéndose diferencias significativas en función del compuesto.

En la segunda parte de esta investigación se usó un cromatógrafo de líquidos acoplado a un espectrómetro de masas de alta resolución (LC–QTOF) programado en el modo de resolución MS/MS para la identificación de otros compuestos presentes en las hojas de estevia. Algunas de las familias de compuestos más relevantes identificadas en las muestras fueron: ácido quínico, cafeico y derivados, flavonoides, glicósidos de esteviol, aminoácidos, ácidos grasos, glicerolípidos y amidas de ácidos grasos. Los ácidos cafeoilquínicos proporcionaron picos cromatográficos de una gran intensidad, equiparable a la de los glicósidos de esteviol, por lo que su alta concentración puede aportar una significativa capacidad antioxidante a la estevia y a sus productos. Además de los glicósidos de esteviol conocidos hasta la fecha, se identificaron nuevos compuestos pertenecientes a esta familia y en función de su patrón de fragmentación se consiguió proponer y justificar su estructura. Por último, se propuso una estrategia para la identificación de las amidas de ácidos grasos, ya que son compuestos poco estudiados hasta la fecha y no están contemplados en las bases de datos de espectrometría de masas.

Agradecimientos: Se agradece a la empresa Vitrosur S.L. el suministro de muestras de estevia.

XV REUNIÓN DEL GRUPO REGIONAL ANDALUZ DE LA SOCIEDAD ESPAÑOLA DE QUÍMICA ANALÍTICA

Almería 30 de Junio – 1 de Julio de 2016

CERTIFICADO DE PARTICIPACIÓN

El Comité Organizador certifica que:

M. Molina Calle, F. Priego Capote, M. D. Luque de Castro

Han presentado la Comunicación ORAL "**Estevia: Cuantificación de glicósidos de esteviol mediante LC-QqQ e identificación de otros compuestos mediante LC-QTOF**" en la XV Reunión del Grupo Regional Andaluz de la Sociedad Española de Química Analítica, celebrada en Almería el 30 de Junio y 1 de Julio de 2016.

Y para que así conste, se expide el presente certificado

En Almería, a 1 de Julio de 2016

Dra. Antonia Garrido Frenich

Presidenta del Comité Organizador

V Congreso Científico de Investigadores en Formación de la Universidad de Córdoba,
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Córdoba, 30 de noviembre, 1 y 2 de diciembre de 2016
En el Rectorado de la Universidad de Córdoba
Avda. Medina Azahara 5

Caracterización de ajo fresco y fermentado mediante LC−QTOF MS/MS: cambios composicionales durante la fermentación

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Summary

Despite black (fermented) garlic is gaining popularity in the cuisine, few studies have been devoted to in-depth profiling characterization of this product from fresh garlic. In the research here presented, polar extracts from fresh and black garlic of three garlic varieties cultivated in the South of Spain were characterized using liquid chromatography with tandem mass spectrometry in high resolution mode with a quadrupole time-of-flight (QTOF) analyzer. One hundred and one compounds were tentatively identified and classified in groups as a function of the given family: amino acids and derivatives, organosulfur compounds, saccharides and derivatives, lipids and derivatives, and others. The composition of three garlic varieties (White, Purple and Chinese) was compared and the differences among them evaluated. Then, fresh and black garlic were compared to determine the compounds that are exclusive to each one and also the compounds with significantly different concentration.

Resumen

A pesar de que el ajo negro tiene cada vez más popularidad en la cocina, solamente unos pocos estudios se han centrado en la caracterización de su composición en profundidad. En la investigación presentada se caracterizaron los extractos polares de ajo fresco y negro mediante cromatografía líquida con espectrometría de masas en tándem usando el modo de alta resolución con un analizador cuadrupolo tiempo de vuelo (QTOF). Se identificaron un total de ciento un compuestos, que se clasificaron en función de la familia a la que pertenecen: aminoácidos y derivados, compuestos organosulfurados, sacáridos y derivados, lípidos y derivados, y otros. Se comparó la composición de tres variedades de ajo fresco (Blanco, Morado y Chino) y se evaluaron las diferencias entre ellas. Asimismo, se

compararon ajo fresco y negro para establecer tanto los compuestos exclusivos de cada uno, como los que presentaron una concentración significativamente diferente.

Introducción

El ajo se usa ampliamente como sazonador en las cocinas de todo el mundo, sobre todo en Asia, África y Europa. Además, numerosos estudios le han atribuido propiedades beneficiosas para el organismo, como, por ejemplo, efectos beneficiosos contra enfermedades cardiovasculares [1] y protectores contra el cáncer [2,3]. Actualmente el ajo fermentado o ajo negro está ganando popularidad, especialmente en la cocina de vanguardia. La producción de ajo negro se basa en un proceso térmico del ajo fresco durante 1−3 meses a una temperatura entre 60 y 80 ºC, unas condiciones de humedad controladas y sin aditivos. Durante este proceso tiene lugar una serie de reacciones, como la reacción de Maillard, que cambian tanto su composición como su aroma y aspecto, dándole su color negro característico.

Numerosos científicos han estudiado la composición del ajo fresco, como se muestra en la revisión de Lanzotti, donde se detallan los compuestos identificados en ajo fresco y los métodos analíticos utilizados para su identificación y cuantificación [4]. Entre estos compuestos cabe destacar los organosulfurados, compuestos característicos de la familia *Alluim* (cebolla, ajo y puerro son los más conocidos en esta familia) y a los que se les atribuye la mayoría de las propiedades beneficiosas del ajo. Sin embargo, los estudios sobre ajo negro llevados a cabo hasta la fecha son más limitados ya que se centran principalmente en su capacidad antioxidante y contenido en compuestos fenólicos [5]. El estudio más completo sobre ajo negro es el llevaron a cabo Montaño et al., en el que cuantificaron aminoácidos y algunas vitaminas en ajo fresco y negro [6].

Por ello, los objetivos de esta investigación fueron: caracterizar el perfil polar del ajo fresco y negro; realizar una comparación para discernir los compuestos exclusivos de cada uno de ellos y los que presentan una diferencia significativa en su concentración; así como analizar la evolución de dichos compuestos durante el proceso de fermentación del ajo negro.

Metodología

Los dientes de ajo de cada una de las muestras, provistos por La Abuela Carmen S.A., se cortaron en trozos y los compuestos polares se extrajeron mediante maceración del ajo

cortado con metanol–agua 50:50 (v/v) durante 30 min. Se filtraron los extractos obtenidos y se analizaron mediante cromatografía líquida con espectrometría de masas en tándem usando el modo de alta resolución con un analizador QTOF. La información obtenida en modo MS/MS se comparó con la de diferentes bases de datos como METLIN (http://metlin.scripps.edu/), Food Database (http://foodb.ca/), MassBank (http://www.massbank.jp/) and MetFrag (http://msbi.ipb-halle.de/MetFrag/) para la identificación de los compuestos.

Resultados

Se identificaron un total de 101 compuestos, con un error por debajo de 5 ppm, y se clasificaron en aminoácidos y derivados, compuestos organosulfurados, sacáridos y derivados, lípidos y derivados, y otros. Todos los aminoácidos identificados en las muestras fueron confirmados mediante el análisis de patrones de estos compuestos.

En ajo fresco se detectaron 82 de estos compuestos de los cuales se identificaron los que presentaban una concentración significativamente diferente entre las variedades de ajo fresco estudiadas (Blanco, Morado y Chino). Entre ellos cabe destacar los aminoácidos valina, asparagina, lisina y arginina, que presentaron una concentración mayor en la variedad de ajo Morado, mientras que la concentración de triptófano fue mayor en la variedad Chino.

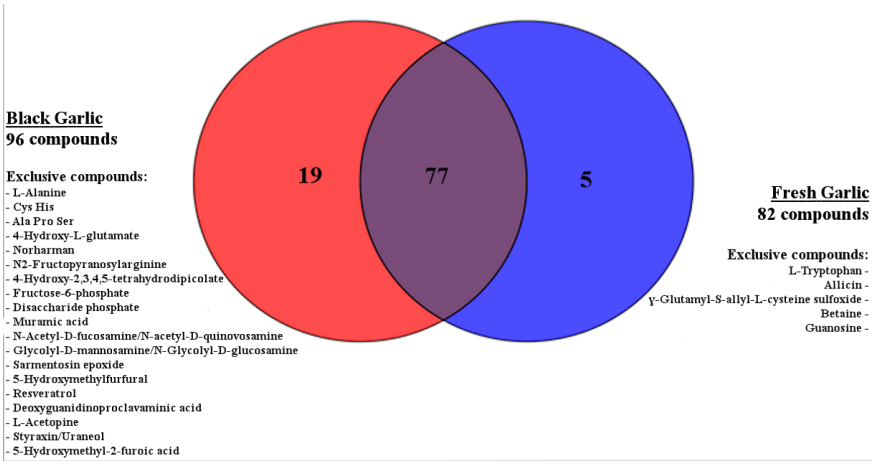

Figura 1. Diagrama de Venn del número de compuestos identificados en ajo negro frente al ajo fresco y los compuestos exclusivos de cada tipo de muestras.

Se compararon las muestras de ajo fresco con las de ajo negro para determinar los compuestos exclusivos de cada uno de ellos (ver Figura 1). 19 compuestos fueron exclusivos de ajo negro, entre los que destacan la alanina y el norharman, un derivado del triptófano; mientras que sólo 5 compuestos fueron exclusivos del ajo fresco: triptófano, alicina, sulfóxido de ɣ-glutamil-S-alil-L-cisteína, betaína y guanosina. Estas diferencias composicionales indican que el triptófano se transforma en norharman durante el proceso de fermentación mediante la condensación del triptófano con piruvato. También cabe destacar que la gran mayoría de sacáridos identificados fueron exclusivos del ajo negro, lo que sugiere que se forman por degradación de sacáridos de mayor tamaño en la reacción de Maillard que tiene lugar durante la fermentación.

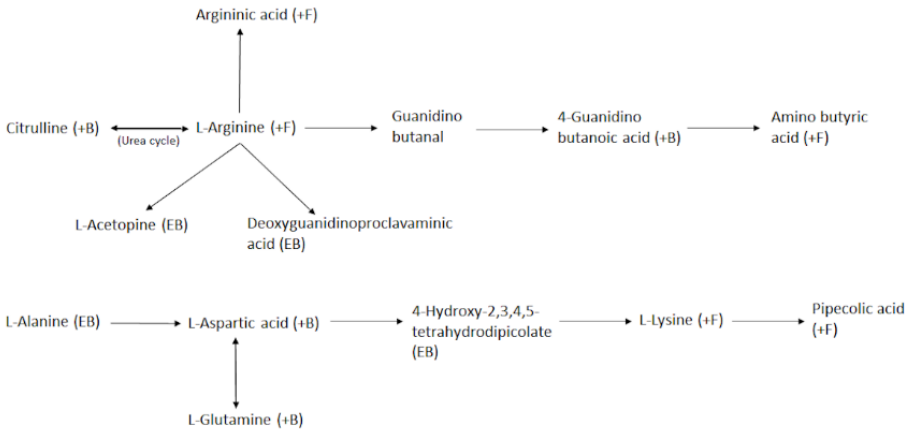

Figura 2. Rutas metabólicas de síntesis y degradación de la arginina, alanina, ácido aspártico y lisina, donde están involucrados algunos de los compuestos identificados. Los compuestos que presentaron una mayor concentración en ajo fresco y negro están marcados con (+F) y (+B), respectivamente, mientras que (EB) corresponde a compuestos detectados exclusivamente en ajo negro.

Se aplicó un test de Volcano para determinar los compuestos con concentración significativamente diferente de entre los 77 compuestos comunes a ajo fresco y negro. Los aminoácidos y derivados fueron el grupo con mayores diferencias entre ambas muestras. La lisina y la arginina presentaron una concentración muy superior en ajo fresco, mientras que la glutamina, fenilalanina y el ácido aspártico resultaron estar mucho más concentrados en ajo negro. Este comportamiento, junto con el de otros derivados de aminoácidos,

permitió determinar las rutas de síntesis y degradación de los aminoácidos afectadas por el proceso de fermentación del ajo negro (ver Figura 2). En cuanto a los compuestos organosulfurados, la mayoría de los que presentaron diferencias significativas en su concentración estaban más concentrados en ajo fresco. Por último, 9 sacáridos y derivados presentaron una concentración significativamente mayor en ajo negro, lo que refuerza la teoría de que la mayor parte de los compuestos identificados en este grupo se generan durante la fermentación por degradación de sacáridos de cadena larga.

Conclusiones

Los resultados obtenidos han permitido discernir entre distintas variedades de ajo fresco, obteniendo una mayor diferencia entre ellas en el perfil de aminoácidos y derivados. También se han estudiado los perfiles composicionales de ajo fresco y ajo negro, quedando de manifiesto claras diferencias cualitativas entre ellos. Además, se estudiaron las diferencias cuantitativas entre ellos, quedando de manifiesto que la mayoría de compuestos identificados en este trabajo presentaron diferencias significativas en su concentración al comparar ambos tipos de ajo. Por último, los resultados permitieron relacionar las diferencias cualitativas y cuantitativas entre ambas muestras con las rutas metabólicas y reacciones químicas implicadas en la producción del ajo negro.

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UNIVERSIDAD D CÓRDOBA

La Vicerrectora de Estudios de Postgrado y Formación Continua de la Universidad de Córdoba

ACREDITA que:

MOLINA CALLE, MARÍA

ha presentado en la modalidad de **COMUNICACIÓN ORAL** el trabajo que lleva por título :

CARACTERIZACIÓN DE AJO FRESCO Y FERMENTADO MEDIANTE LC-QTOF MS/MS: CAMBIOS COMPOSICIONALES DURANTE LA FERMENTACIÓN

en el **V Congreso Científico de Investigadores en Formación de la Universidad de Córdoba,** organizado por la Escuela de Doctorado de la Universidad de Córdoba, celebrado en Córdoba los días 30 de Noviembre y 1 de Diciembre de 2016.

Y para que así conste, se expide y firma este certificado en Córdoba, a 1 de diciembre de 2016

Fdo: JULIETA MÉRIDA GARCÍA

Vicerrectora de Estudios de Postgrado y Formación Continua de la Universidad de Córdoba

ANEXO III

Otras actividades formativas

- Actividad Paseo por la Ciencia.
  Organizado por: Asociación Profesorado de Córdoba por la Cultura Científica (APCCC). Córdoba (España), 2014.
  Tipo de formación: Actividades divulgativas

- V Encuentro sobre Nanociencia y Nanotecnología de Investigadores Andaluces.
  Organizado por: Instituto Universitario de Investigación en Química Fina y Nanoquímica (IUIQFN). Córdoba (España), 2015.
  Tipo de formación: Asistencia a congreso

- Jornada de Bioestadística para Periodistas y Comunicadores.
  Organizado por: Fundación Dr. Antonio Esteve y Asociación Española de Comunicación Científica. Córdoba (España), 2016.
  Tipo de formación: Participación en jornadas de formación

- Curso Nanotecnología para Todos.
  Organizado por: Departamento de Química Analítica. Córdoba (España), 2016.
  Tipo de formación: Curso formativo

D. Rafael Enriquez Centella, con DNI 30485653G, secretario de la Asociación Profesorado de Córdoba por la Cultura Científica (APCCC), con sede social en Córdoba, C/ Doña Berenguela s/n e inscrita en el registro de asociaciones de Andalucía con el número 14/1/06230

CERTIFICO

Que Dª. María Molina Calle con DNI 53594555R, investigadora del Departamento de Química Analítica de la Facultad de Ciencias (Universidad de Córdoba), ha participado en el **Paseo por la Ciencia**, una actividad de 10 horas de duración, organizada por esta asociación el día 5 de abril de 2014.

En Córdoba, a 5 de abril de 2014.

Fdo: Rafael Enriquez Centella

VºBº: María Ropero Romero
PRESIDENTA DE LA APCCC

VºBº: Manuel Blázquez Ruiz
DECANO DE LA FACULTAD DE CIENCIAS

V Workshop
NANOUCO

NANOUCO V

Encuentro sobre Nanociencia y Nanotecnología de Investigadores y Tecnólogos Andaluces

Córdoba, 5 y 6 de Febrero de 2015

El Comité Científico del V Encuentro sobre Nanociencia y Nanotecnología de Investigadores Andaluces, celebrado en Córdoba los días 5 y 6 de febrero de 2015,

HACE CONSTAR

Que Dª María Molina Calle ha asistido al desarrollo del citado evento.

Córdoba, 6 de febrero de 2015.

Fdo: María Dolores Sicilia Criado
Presidenta del Comité Científico

Fdo: Rafael Lucena Rodríguez
Secretario del Comité Científico

FUNDACIÓN DR. ANTONIO ESTEVE

AECC Asociación Española de Comunicación Científica

Córdoba, 26 de octubre de 2016

La **Fundación Dr. Antonio Esteve** y la **Asociación Española de Comunicación Científica**
certifican que

María Molina Calle

ha participado en la

Jornada de bioestadística para periodistas y comunicadores

celebrada en la **Universidad de Córdoba**
el **26 de octubre de 2016.**

Fèlix Bosch
FDAE

Gonzalo Casino
AECC

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Miguel Valcárcel, Catedrático del Departamento de Química Analítica de la Universidad de Córdoba, con DNI 28274746H,

En calidad de:

Director Académico del curso de Formación del Profesorado titulado "Nanotecnología para todos", de 2 créditos ECTS, celebrado en Córdoba los días 17, 18, 19 de octubre de 2016 y 7, 8 de noviembre de 2016,

HACE CONSTAR:

Que Dª MARÍA MOLINA CALLE (DNI: 53594555R) ha asistido con aprovechamiento al citado curso, en el que dentro del Bloque IV del mismo ha realizado una presentación sobre la temática "Impacto de la Nanotecnología en la Metabolómica".

Córdoba, 11 de noviembre de 2016.

Miguel Valcárcel Cases

ABREVIATURAS

ABTS, 2,2'-azinobis-(3-etilbeztiazolin-6-sulfonato)

ACN, acetonitrilo

ANOVA, análisis de varianza

APCI, ionización química a presión atmosférica

ARNm, ARN mensajero

ASE, extracción por solvente acelerada

BPC, cromatograma de pico base

BSTFA, *N,O*-bis(trimetilsilil)trifluoroaceteamida

CA, análisis de clústeres

CE, electroforesis capilar

CI, ionización química

DAD, detector de diodos en fila

DIMS, espectrometría de masas con inyección directa

DPPH, 2,2-difenil-1-picrilhidrazilo

EFSA, Autoridad Europea en Seguridad Alimentaria

EI, ionización por impacto electrónico

EIC, cromatograma de un único ión

ESI, ionización por electrospray

EU, Unión Europea

FA, ácido fórmico

FAO, Organización de las Naciones Unidas para la Alimentación y la Agricultura

FID, detector de ionización en llama

FLD, detector de fluorescencia

FRAP, reducción del ion férrico complejado

F−C, Folin−Ciocalteu

GC, cromatografía de gases

GH, invernadero

HMDB, base de datos del metaboloma humano

HPLC, cromatografía líquida de alta resolución

HPSE, extracción con solvente a alta presión

HPTLC, cromatografía en capa fina de alta resolución

HS, espacio de cabeza

IMIBIC, Instituto Maimónides de Investigación Biomédica de Córdoba

IR, infrarrojo

IT, trampa de iones

KEGG, enciclopedia de Genes and Genomas de Kyoto

LAB, laboratorio

LC, cromatografía de líquidos

LLE, extracción líquido–líquido

LOD, límite de detección

LOQ, límite de cuantificación

NMR, resonancia magnética nuclear

MAE, extracción asistida por microondas

METLIN, base de datos de metabolitos obtenida mediante MS en tándem

MeOH, metanol

MF, entidad molecular

MPP, "Mass Profile Professional"

MS, espectrometría de masas

MS^n, espectrometría de masas con fragmentación múltiple

MS/MS, espectrometría de masas en tándem

MSPD, dispersión de la matriz en fase sólida

MW, microondas

m/z, relación masa carga

NIST, Instituto Nacional de Estándares y Tecnología

ORAC, capacidad de absorción de radicales de oxígeno

PAH, hidrocarburos aromáticos policíclicos

PCA, análisis de componentes principales

PFE, extracción con fluidos presurizados

PHSE, extracción con solvente caliente presurizado

PLS, análisis de mínimos cuadrados parciales

PLS-DA, análisis discriminante de mínimos cuadrados parciales

QqQ, triple cuadrupolo

QTOF, cuadrupolo acoplado a tiempo de vuelo

RP, fase reversa

RSD, desviación estándar relativa

RT, tiempo de retención

SF, fluido supercrítico

SFC, cromatografía de fluidos supercríticos

SFE, extracción con fluido supercrítico

SHLE, extracción con líquido sobrecalentado

SHSE, extracción con solvente sobrecalentado

SIMCA, "soft independent modeling class analogy"

SPE, extracción en fase sólida

SPME, microextracción en fase sólida

SRM, monitorización de reacciones seleccionadas

SSE, extracción con solvente subcrítico

TAC, contenido total de antocianinas

TAI, índice total de antocianinas

TEAC, capacidad antioxidante equivalente a TROLOX

TIC, cromatograma de iones totales

TOF, tiempo de vuelo

TPI, índice total de fenoles

TROLOX, 6-hidroxi-2,5,7,8-tetrametilcroman-2-carboxílico

UPLC, cromatografía líquida de ultra alta resolución

US, ultrasonidos

USAE, extracción asistida por ultrasonidos

USAEE, emulsión y extracción simultáneas asistidas por ultrasonidos

UV, ultravioleta

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